INTERACTIONS BETWEEN METAL AND DROUGHT STRESSORS ON PLANT WATER RELATIONSHIPS AND THEIR EFFECTS AT ECOSYSTEM LEVEL

by

Fallon M. Tanentzap

A thesis submitted in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy (Ph.D.) in Boreal Ecology

The Faculty of Graduate Studies
Laurentian University
Sudbury, Ontario, Canada

THESIS DEFENCE COMMITTEE/COMITÉ DE SOUTENANCE DE THÈSE
Laurentian Université/Université Laurentienne
Faculty of Graduate Studies/Faculté des études supérieures

Title of Thesis
Titre de la thèse INTERACTIONS BETWEEN METAL AND DROUGHT STRESSORS ON PLANT WATER RELATIONSHIPS AND THEIR EFFECTS AT ECOSYSTEM LEVEL

Name of Candidate
Nom du candidat Tanentzap, Fallon

Degree
Diplôme Doctor of Philosophy

Department/Program
Département/Programme Boreal Ecology

Date of Defence
Date de la soutenance March 24, 2015

APPROVED/APPROUVÉ

Thesis Examiners/Examinateurs de thèse:

Dr. Peter Ryser
(Supervisor/Directeur de thèse)

Dr. Gerard Courtin
(Committee member/Membre du comité)

Dr. Glen Watson
(Committee member/Membre du comité)

Dr. Stephen Monet
(Committee member/Membre du comité)

Dr. Ewa Cholewa
(Committee member/Membre du comité)

Dr. Neil Emery
(Committee member/Membre du comité)

Dr. Rainer Schulin
(External Examiner/Examinateur externe)

Dr. Graeme Spiers
(Internal Examiner/Examinateur interne)

Approved for the Faculty of Graduate Studies
Approuvé pour la Faculté des études supérieures
Dr. David Lesbarrères
M. David Lesbarrères
Acting Dean, Faculty of Graduate Studies
Doyen intérimaire, Faculté des études supérieures

ACCESSIBILITY CLAUSE AND PERMISSION TO USE

ABSTRACT

Heavy metals influence plant traits that are relevant to its internal water status. Therefore, knowledge of metal effects on a plant's ability to tolerate other stresses such as drought is important to ensure sustainability of restoration efforts in contaminated landscapes, often prone to drought. The aim of this thesis was to gain understanding how heavy metal contamination influences plant-water relationships and how this interacts with effects of a simultaneous drought. Levels ranging from organs to ecosystem processes were investigated. Chapter 1 briefly reviews published studies on interactions between metal and drought stress and highlights potential gaps in literature. Chapter 2 presents results of a 2-year outdoor lysimeter study on the effects of Cu-Ni toxicity on plant-water relationships using saplings of *Acer rubrum, Betula papyrifera*, and *Quercus rubra*. The study shows that under elevated metal levels the plants use less water but suffer drought symptoms even if there is water available. Chapter 3 investigates, using *B. papyrifera,* whether such metal effects on water use are a result of short-term exposure of roots on their water uptake capacity, or due to long-term structural damage e.g. on hydraulic architecture. Both effects were found, but were smaller than hypothesized. In Chapter 4 influence of metals on plant's vulnerability to xylem embolisms in *Acer rubrum* saplings is found to be higher in the metal contaminated region of Sudbury, Ontario, compared to surrounding uncontaminated regions. Chapter 5 establishes guidelines and limitations for transporting leaves from field to laboratory when measuring leaf relative water content, a method that is important for studying plant water relations in the field. Finally, I conclude with a general overall summary of results and proposed suggestions for reclamation projects.

Keywords: heavy metals, drought, plant-water relationships, trees

ACKNOWLEDGEMENTS

Firstly, I would like to acknowledge my supervisor Dr. Peter Ryser for his continual support, encouragement and for all the opportunities throughout ALL of the years. Thank you.

Secondly, I would like to thank my committee members Dr. Gerard Courtin, for always making me think outside the box and giving extremely helpful advice on the project; as well as Dr. Stephen Monet and Glen Watson for providing helpful information and participating in Industry Research collaboration, and for all of the extra funding and providing helping hands and equipment to make a project of this size possible. Also, a big thank you to Dr. Ewa Cholewa and Dr. Neil Emery for taking on this project at last minute. Everyone's advice and guidance was appreciated.

Thank you family and friends for your continual love and encouragement to keep me going full steam ahead around every milestone. I would like to further acknowledge my brother, Logan Kirkey, for jumping to my rescue every time I needed any extra assistance including but not limited to all of the heavy lifting and construction of all the apparatuses that I needed throughout the 4 years; both of my parents: my Dad (Dan Kirkey) for his daily support and for the construction of the large lysimeter balance; and my Mom (Shirley Ann Kirkey) for helping me keep both myself and the trees alive on many occasions. A BIG thank you to my husband, Andrew Tanentzap, for all of the constant motivation to keep on going and help editing my thesis including helping me learn the basics of R and being there for any advice I needed.

Thank you to all others that made this project possible: Paul and Carol at Holla's Greenhouse and Nursery for building and maintaining the greenhouse, Dr. Uwe Hacke at the University of Alberta for showing me the methods for measuring embolism resistance in tree stems, Alexandra Stempel for all of the data collection in the RWC chapter 5 and Kierann Santala for listening to me on our many jogs and for having amazing advice for curing writers block. Last but not least I would like to thank my poodle - Moose, you have been there from the beginning my loyal little side kick.

This thesis was funded by the Collaborative Research and Development grant from the Natural Sciences and Engineering Research Council of Canada (NSERC) with Vale Canada Ltd. and the Centre for Excellence in Mining Innovation (CRDPJ 372568-08), and by Laurentian University Faculty of Graduate Studies. Additional in-kind support was received from Vale Canada Ltd.

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GENERAL INTRODUCTION

Plant water relationships are influenced by soil water availability, water uptake by roots, and transportation through xylem and loss through transpiration in leaves. Maintenance of an adequate water status requires that all these processes work within given limits. Metal stress is known to influence these processes. Experiments in this Ph.D. thesis investigated the effect of substrate contamination by a mixture of heavy metals, mainly copper and nickel, on the plants ability to regulate these processes under drought, to maintain growth and avoid mortality.

The overall aim of this thesis is to gain a greater understanding of the effects heavy metal contamination has on plant-water relationships. The focus is on the interaction between metal and drought stress, i.e., how exposure to metals influence a plant's tolerance of drought. The question is addressed at different organizational levels, ranging from leaves and roots to whole plants and ecosystem level processes. The different aspects of this topic are addressed in the following chapters:

Chapter 1 – Literature review to assess the current state of knowledge on potential drought – metal interactions.

Charter 2 – Metal effects on plant water relations at ecosystem level and the feedback on soil water availability.

Chapter 3 – Effects of metals on water uptake and transpiration

Chapter 4 – Effects of metals on failure of transportation system: embolism.

Chapter 5 – Methodical constraints of assessing leaf relative water content in the field.

My hypothesis is that metal contamination damages the mechanisms that plants use to regulate their water status and hence increases their vulnerability to drought.

Approach:

The main experiment was a two-year lysimeter study of metal and drought-stress in factorial design. In this experiment, starting in the spring of 2011, I examined the plant-water relationships in three broad-leaf tree species (*Acer rubrum, Betula papyrifera* and *Quercus rubra*) including their growth substrate outdoors but protected with a plastic canopy in Val Caron, Ontario. Results from this study led to the development of two complementary studies with selected species to further investigate factors leading to the observed reduction of water use under metal stress, and to investigate whether the observed effects would lead to plant vulnerability to embolism and mortality in a field situation. Moreover, one experiment was conducted to test the accuracy of a method used to assess plant water status.

Significance:

This project will make an important contribution to the understanding of the influence of metal contamination on plants responses to drought. In order to ensure future success of reclamation projects it is imperative to better understand what the plants ecophysiological dynamics at different organizational levels under simultaneous influence of multiple stressors, in this case metal contamination and drought, in order to make further recommendations.

CHAPTER 1

INFLUENCE OF METAL CONTAMINATION ON PLANTS' RESPONSES TO DROUGHT: A REVIEW FROM MOLECULES TO WHOLE PLANTS

1.1 Abstract:

In their review on the effects of heavy metals on plant-water relationships Poschenrieder and Barceló (2004) state that the topic had attracted relatively little attention considering its importance. Studies on combined effects of these stresses remain rare, but there has been considerable progress since 2004. This review gives a synopsis of literature on the influence of metal contamination on plant-water relationships at different organization levels and attempts to build a link towards understanding how simultaneous metals and drought stress affect water relations at the ecosystem level. This review addresses topics such as root water uptake, membrane permeability, xylem and hydraulic conductance, and summarizes the relationship between transpiration and relative water content (RWC) as affected by metals. The general trend observed is that simultaneous metal and drought stresses additively increase water stress in plants. Metal treatments reduce root length, decrease xylem size and specific conductivity, and increase vessel blockage, resulting in an overall decrease in hydraulic conductivity and resistance to embolism. Transpiration rates are mostly reduced, but the effect depends on plant species, duration of exposure and the amount of metals. The results show that the relationships are complex and that more research is needed to identify the influence of metal contamination on plants responses to drought.

1.2 Introduction:

This review summarizes current literature on the influence of metal contamination on plant-water relationships from molecular to whole plant levels in order to understand the combined effects of these two stresses on plants and ecosystem processes better, and to identify gaps in our current knowledge. I compiled results from the growing collection of studies investigating the effect of heavy metal stress on plants' responses to drought at different organizational levels, focusing on studies that have been published since the comprehensive reviews of Barceló and Poschenrieder (1990) and Poschenrieder and Barceló (2004), but not excluding older, relevant literature. This review addresses new literature on topics addressed by Barceló and Poschenrieder (1990) such as root water uptake, membrane permeability, and xylem and hydraulic conductance, but also summarizes the known aspects of the relationship between transpiration and relative water content (RWC), by focusing on simultaneous metal and drought stress. Understanding how metal contamination influences the physiology and morphology of plants in the context of their ability to respond to other abiotic stressors such as drought can aid projects aiming to mitigate and rehabilitate land affected by these stressors.

Heavy metal as a term is still being criticized as inaccurate and meaningless (Nieboer and Richardson, 1980; Duffus. 2002), but it remains widely used within the field of environmental pollution and plant sciences (Duffus, 2002; Appenroth, 2010). The term heavy metals was originally defined as metals with a specific gravity above a certain threshold, such as 4 (Bjerrum, 1936; Nieboer and Richardson, 1980; Prasad, 2004; Duffus, 2002), but this threshold has not been consistently used making it unreliable as a definition. Also, not all metals with high specific gravity have similar physiological effects (Nieboer and Richardson, 1980). However, the continuing use of the term 'heavy

metals' in the field of plant sciences and restoration can be justified by the fact that despite decades of efforts no other term has caught on (Duffus, 2002). The term is well known with practitioners and public, and is now more commonly associated with metals occurring at toxic levels, especially those extracted and used by humans, and potentially contaminating large areas causing environmental problems (Shaw, 2004). Toxic levels of such metals inhibit plant growth or reduce plant fitness, but it is important to note that even though these metals cause stress responses at higher concentrations, many of them are also essential micronutrients and are required at low concentrations (Prasad, 2004).

Soils that have been impacted by anthropogenic sources of heavy metals often have poor water holding capacity, which has been frequently considered as a reason for poor success of re-establishment of primary vegetation on such disturbed landscapes (James and Courtin, 1985; Barceló and Poschenrieder, 1990). Despite the common co-occurrence of these stresses, studies into the interactions between drought and metal contamination are rare (Poschenrieder and Barceló, 2004; Eränen and Kozlov, 2009), and the limited amount of literature about the topic has been described as 'frustrating' by Disante *et al.* (2010). The published results from such studies are extremely variable and depend on exposure time, amount of metal, and plant species under investigation. The influence of drought on soil properties and metal availability must also be considered because drought can cause changes in the substrate. This has been observed in acid sulphate soils in Australia where the amounts of bioavailable metals increased after the soil has been dried out and re-watered (Simpson *et al*., 2010). Kawahigash *et al.* (2008) also show experimentally that, after drying, soil pH decreased causing a spike in bioavailable metals in the substrate over time. Heavy metal availability is also enhanced by anthropogenic soil acidification, e.g., caused by sulphuric dioxide emissions and

deposition on thin soils with low buffering capacity (Hutchinson and Whitby, 1977; Tangahu *et al*., 2011).

Plant physiological and morphological responses to stresses generally alleviate the effects (Mooney *et al.,* 1991; Lambers *et al.,* 2008). Different stresses are studied mostly individually, but it is known that responses to a combination of two stressors cannot necessarily be deduced from the responses to the individual stresses (Rivero *et al*., 2014). Responses to metal contamination and drought have mostly been investigated separately (Barceló and Poschenrieder, 1990), which may limit the validity of conclusions about responses in a multi-stress environment (Mikkelsen *et al.,* 2008). For example, plants respond to drought by increasing root length in order to maximize water uptake, and by closing stomata to reduce water loss (Slayter, 1967). However, metal contamination has been shown to stunt root elongation and has variable effects on stomatal movement (Pochenrieder and Barceló, 2004). Thus, metals and drought can have conflicting effects on plants, and the net effect in plants exposed to both metal and drought stresses may produce shorter root systems that increases their susceptibility to drought and threatens their survival.

The objectives of this review are to address the influence of heavy metals on root water uptake, membrane permeability, xylem and hydraulic conductance, and summarize the relationship between transpiration and RWC under simultaneous drought treatment. By understanding the role drought plays on toxic levels of metals in plant morphology and physiological process it can improve vegetation restoration processes as well as help long-term monitoring plans in areas prone to drought.

1.3 Simultaneous Heavy Metals and Drought on Root Water Uptake and Membrane Permeability:

Root elongation has frequently been used as an indicator of plant response to metals (Wilkins, 1957; Wong and Bradshaw, 1982). Metal stress in roots has been shown to inhibit elongation, creating short (Kahle, 1993), thick roots over time (Ryser and Emerson, 2007). The reduced elongation may be a result of impeded cell elongation as a result of a lower membrane permeability to water (Hukin *et al*., 2002). Arduini *et al*. (1995) found that root cell elongation was reduced by 38.7% in *Pinus pinea* due to 1 μM of Cu exposure over a 4 weeks period, which also increased internal cell wall lignification, but did not influence cell division. These authors further suggested that increased Cu exposure reduced internal water status by decreasing root electric potential, due to metals binding around membrane of the root tip cells. Disante *et al.* (2014) found a decrease in root biomass of *Quercus suber* seedlings exposed for 5 months to four levels of Zn, at either high or low watering, but they also observed that drought caused a 33% increase in root length in the highest Zn treatment, compared to the non-drought treatment. These studies suggest that combined effects of drought and metal contamination on root length are not necessarily predictable from the effects of single stresses.

Aquaporins are transmembrane proteins that regulate root water uptake and hence affect how their water status responds to stress (Maurel and Chrispeels, 2001). Presence of Hg has been shown to inhibit water transport through root membranes, an effect that is reversible (Maggio and Joly, 1995; Wan and Zwiazek, 1999). It has also been shown that *Pisum sativum* upregulates aquaporin expression in response to exposure to Hg, probably to compensate for the blocked aquaporins (Beaudette *et al*., 2007). So far there are only a

limited number of studies that have addressed the role of plant aquaporins in relation to other heavy metal contamination. Kholodova *et al*. (2011) have shown that aquaporin gene expression was reduced over 50% in roots and leaf cells of *Mesembryanthemum crystallinum* after only 24 h of exposure to 25 or 50 μM Cu. Przedpelska-Wasowicz and Wierzbicka (2011) have described that aquaporin blockage may occur within as fast as ten minutes after metal exposure and the effect magnitude depends on the type of metal and the degree of metal toxicity. In one case, an aquaporin gene of the metal-accumulator *Brassica juncea,* expression of which is up-regulated in response to exposure to metals, was introduced in tobacco and it was shown to enhance the resistance of tobacco root growth to Cd and helped the plant to maintain its internal water status under Cd exposure (Zhang *et al.,* 2008).

1.4 Simultaneous Heavy Metals and Drought on Xylem and Hydraulic Conductance:

There is evidence that heavy metals also influence plant water transport through xylem, some experimental studies indicating that there might be a reduction and even an interruption in longitudinal water transport within plants caused by exposure to metals. Lamoreaux and Chaney (1977) and de Silva *et al*. (2012) found blockages within the xylem cells, including cellular debris in response to Cd exposure of *Acer saccharinum*, and in response to Ni and Cu exposure of *Acer rubrum,* respectively. A possible explanation for the blockage in the xylem elements may be due to chelated metals causing an internal build-up of H_2SO_4 that hastens cell breakdown and membrane leakages (Gajewska *et al.,* 2012). Also, oxidative damage by heavy metals is known to compromise membrane integrity (Shaw *et al*., 2004). The damaged membranes may lead

to disruption of autolysis processes during xylem maturation and to the observed cellular debris (Bollhöner *et al.,* 2012). Furthermore, metals also reduce xylem conductivity. Santala and Ryser (2009) illustrated that stem diameter was decreased by 41% and the number of xylem radial cells decreased by approximately 50% in *Betula papyrifera* seedlings with exposure to Co, Cu and Ni. de Silva *et al*. (2012) showed that both Cu-Ni in combined contamination and drought additively reduced stem cross sectional area by 57% and root xylem vessel area by 53%, thereby lowering potential hydraulic conductance by 30% within *Acer rubrum* seedlings. Decreasing xylem size, increasing vessel blockage and membrane leakage are strong indicators that metals may reduce water transport capacity in a plant.

Reduced hydraulic conductivity due to smaller vessel sizes are considered to be a tradeoff to increase hydraulic safety (Hacke *et al*., 2006). Hence, if metal contamination decreases xylem size then theoretically it should also increase embolism resistance. However, it has also been found that small vessels may aggravate water stress when evaporative demand is high (Maherali and De Lucia, 2000). Tanentzap and Ryser (2015) found a 14% decrease in stem hydraulic conductance, but also observed a 40% higher rate of embolism in field-grown *Acer rubrum* saplings from a metal-contaminated landscape, compared to saplings from an uncontaminated region. Increased vulnerability to embolisms may be the cause of stem and branch dieback in trees within metal-contaminated landscapes, that have been regularly observed (James and Courtin, 1987; Eränen *et al*., 2009). Watmough (2010) shows that in sugar maple forests (*Acer saccharum*) canopy decline is associated with high levels of Cd, Zn, Cu and Pb in the surrounding soil. Overall, the longitudinal transport of water within trees seems to be

severely impacted by soil metal contamination thus potentially reducing forest canopy success by reducing embolism resistance.

1.5 Influence of Drought on Leaf Heavy Metal Concentrations:

Leaf metal uptake is frequently reported and of interest especially for applied purposes as metal hyperaccumulator species can be potentially used for phytoremediation purposes (Sarma, 2011). It has been hypothesized that hyperaccumulation of metals in leaves might increase drought tolerance (Boyd and Martens, 1998), but no evidence for this has been found (Whiting *et al*., 2003). However, accumulation in combined metal and drought stress plants is more variable and rarely reported. Increased foliar levels of Ni and Co were found in response to drought in three deciduous tree species (*Acer rubrum, Quercus rubra* and *Betula papyrifera*), but Cu only increased in response to drought, not metal treatment (Chapter 2, this thesis). de Silva *et al.* (2012) found that drought increased foliar Ni, Co and Cu concentrations in *Acer rubrum* leaves grown on contaminated substrate. Drought also increased Zn and Cu leaf concentrations in sewage sludge-treated *Lolium multiflorum*, but for Cd the effect of drought seemed to be the opposite (Pasqual *et al.,* 2004). By contrast, Disante *et al.* (2014) observed the increasing effect of Zn exposure on foliar Zn concentrations in *Quercus suber* to be higher in well-watered plants, compared to plants under a drought treatment. The few reports of metal content in combined factorial experiments have not received the attention they deserve, because they may help explain potential internal leaf processes and the effects that stresses have in combination with different metals at toxic levels on stomatal function, transpiration rate and internal leaf RWC. Metals also interact with each other in their

effect on tissue concentrations. Tani and Barrington (2005) observed that in buckwheat exposure to Cu reduced tissue concentrations for Ni.

1.6 Simultaneous Heavy Metals and Drought on Transpiration and Leaf Relative Water Content:

Studies on the effects of heavy metal contamination on transpiration show results, that are in part contradictory, possibly depending on plant species and metal concentration. Metals have been shown to either increase, decrease or have no effect on transpiration rate (Table 1.1). Cd often decreases transpiration rates (Sheoran e*t al*., 1990; Bishnoi *et al*., 1993; Pandey and Sharma, 2002; Beaudette *et al*., 2007). As a consequence of this, Thakur and Sigh (2012) found an increased foliar temperature by approximately 3°C in plants after 24 hours exposure to Cd. But there also are a few studies showing increasing transpiration in sugar beet due to Cd contamination (Greger and Johansson, 1992). This increase, however, was interpreted as an increased cuticular transpiration, whereas Cd decreased the density of functional stomata and induced their closure. This is in agreement with data reported by Walley and Huerta (2010), who found that Cd increases transpiration, but not photosynthesis. Also some other metals have been described to reduce transpiration rates, such a Ni (Bishnoi *et al.,* 1993) and Co (Chatterjee and Chaterjee, 2000). A combination of metal and drought stresses may create unexpected responses. Transpiration rates of *Quercus suber* decrease along with increasing Zn contamination when plants are well watered, but under dry conditions, intermediate levels of Zn contamination increase the transpiration rates (Disante *et al.,* 2014). Field studies have failed to detect an effect of heavy metals on transpiration, or only a very small effect (Tani and Barrington, 2005)

The aperture status of stomata is the leading regulator of transpiration and plant water loss. Signals such as abscisic acid (ABA) may lead to a stomatal closure, which is achieved by potassium moving out of the guard cells, and the cells losing water, becoming flaccid and closing the pore. Aquaporin densities are important for this water movement in the guard cells (Chaumont and Tyerman, 2014). Heavy metals may also influence potassium movement across cell membranes (Coskun *et al*., 2011). Perfus-Barbeoch *et al.* (2002) suggest that the effect of Cd may be due to calcium channel blockage. Yang *et al.* (2004) investigated the direct influence of Hg, Zn, Cd, and Li on stomatal aperture, showing reduced opening during the day and decreased closure during dark periods. The response of ABA to metal contamination has been identified as metal specific. Hayward *et al.* (2011) have shown that ABA levels spike when exposed to increasing concentration of Cd, but not Ni, which could explain the often-observed reduction in transpiration by exposure to Cd, compared to exposure to Ni (Sheoran *et al.* 1990).

One may conclude, with some exceptions, that metal contamination leads to decreased transpiration rates. One might think that this will improve plant water status, but generally the opposite is the case. Overall, exposure to metals has been shown to decrease leaf relative water content (RWC) (Schlegel *et al*., 1987; Poschenrieder *et al*., 1989; Kholodova *et al*., 2011), and there does not seem to be any general relationship between RWC and transpiration (Table 1.1). Disante *et al*. (2011) found that increasing exposure to Zn reduced the drought-caused decrease in RWC after 6 days of drought. These results indicate that Zn has a different effect on plant water relations than other heavy metals. Some non-metals, such as Se also seem to have similar effects to most of

the metals, by reducing transpiration rates and leaf relative water content, and aggravating the effects of drought (Kostopoulou *et al*., 2010).

Table 1.1 Summary of results of published studies on metal effects on transpiration and leaf relative water content (RWC). The effects are indicated as increased (+), decreased (-), or no difference (/) in transpiration rates or RWC in species studied with various metal contaminations, n/a indicated that RWC was not assessed.

Increased	**Species**	**Metal**	**Transpiration**	**RWC**
Greger and Johansson, 1992	*Beta vulgaris*	Cd	+	n/a
Walley and Huerta, 2010	*Glycine max*	Cd	+	n/a
Either (+/ -) or No Difference				
Schlegel *et a*l., 1987	*Picea abies*	Cd, Hg	-	-
	Picea abies	Zn	/	-
Tani and Barrington, 2003	*Fagopyrum esculentum*	Cu, Zn	/	n/a
Whiting *et al.*, 2003	*Allyssum murale*	Ni, Zn + Drought	/	-
	Thlaspi caerulescens	Ni, Zn + Drought	/	/
Menon *et al.,* 2005	*Salix viminalis*	Co, Cu, Fe, Ni	+	n/a
	Populus tremula	Co, Cu, Fe, Ni	-	n/a
Hermle *et al.,* 2006	*Populus tremula*	Cd, Cu, Pb, Zn	-	n/a
	Salix viminalis	Cd, Cu, Pb, Zn	/	
Mensah *et al.* 2008	*Brassica oleracea*	Cd, Pb	-	n/a
	Daucus carota	Cd, Pb	/	n/a
Decreased				
Bazzaz *et al*., 1974	*Zea mays, Helianthus annuus*	Cd	-	n/a
Kastori *et al.,* 1992	*Helianthus annuus*	Cd, Cu, Pb, Zn	-	-
Perfus-Barbeoch *et al*., 2002	*Arabidopsis thaliana*	Cd	-	/
Menon *et al.* 2007	*Populus tremula, Salix viminalis*	Co, Cu, Fe, Ni	-	n/a
Kostopoulou *et al*. 2010	*Melilotus officinalis*	Si + Drought	-	/
Disante *et al*., 2011	*Quercus suber*	Zn + Drought	-	+
Kholodova *et al*., 2011	*Mesembryanthemum crystallinum*	Cu, Zn	-	-
Thakur and Sigh, 2012	*Glycine max*	Cd	-	-
Disante *etl al*., 2014	*Quercus suber*	Zn+ Drought	-	n/a
Sheoran et al. 1990	*Cajanus cajan*	Cd, Ni	-	-
Bishnoi et al. 1993	*Triticum aestivum*	Cd, Ni	-	-
Chatterjee & Chatterjee 2000	*Brassica oleraceae*	Co, Cr, Cu	-//	-//
Pandey & Sharma 2002	*Brassica oleraceae*	Co, Ni, Cd	-	-

1.7 Simultaneous Heavy Metals and Drought on Ecosystem Processes:

The effect of metals on plant-water relations at a scale larger than individual leaves or plants have rarely been investigated. However, studies connecting plant-level relationships to ecosystem-level are needed to understand metal effects on processes such as water cycling. Menon *et al.* (2005) monitored establishment of understory herbaceous species and woody tree species in model ecosystems consisting of lysimeter pots within open-top chambers over three-years in response to drought and to Cd, Cu, Fe and Ni contamination. Evapotranspiration was reduced by metal contamination, leading to higher soil water potentials, and interpreted mainly as a result of reduced rooting density in the soil. Differences among species responses and changes in species composition over time lead to year-to-year changes in ecosystem water consumption (Menon *et al*., 2005). At the level of individual leaves in this project, transpiration and water use efficiency decreased with the increasing metal contamination, compared to controls (Hermle *et al*. 2006).

Reduced water use by plants in response to metal contamination seems to be leading to more water being retained within the soil (Menon *et al.,* 2005)**.** However, this does not improve the plants' water status, and the effects of combined metal and drought stresses are complex as leaf water status under metal stress is compromised by stunted roots, inhibited aquaporin function, changes and damage in hydraulic architecture, and consequently, a reduced embolism resistance.

1.8 Summary:

Plant-water relationships in response to heavy metal contamination are complex and vary greatly depending on the species of plant and metal. However, the last 10 years

of research generally indicates that the additive effects of simultaneous metal and drought stresses impact plant performance negatively. Similarly to the effect of drought, metals reduce transpiration and plant water use. However, this does not seem to be a result of a reduced stomatal conductance, but of a reduced water acquisition capacity, because leaf RWC is generally reduced by exposure to metals. Metal treatments have been shown to reduce root length, down-regulate aquaporin expression or block it, and have effects on xylem structure, which on the one hand is similar to the effects caused by drought, but on the other hand, makes the xylem more vulnerable to embolisms. Studies investigating simultaneous effects of metals and drought remain rare, and conclusive generalizations are hampered by differences among species and metals. An area which is especially poorly documented is how the different physiological processes influence internal plant water status and how signaling between these processes works. There are still many gaps in the literature and more work is needed. Nevertheless, within the last 10 years there has been a more conclusive understanding on the effect of plant exposure to metals on responses to drought.

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CHAPTER 2

HEAVY METAL STRESS INFLUENCES PLANT WATER RELATIONS AND RESPONSES TO DROUGHT: A TWO-YEAR LYSIMETER STUDY

2.1 Abstract:

The effects of heavy metal contamination on plant drought tolerance were investigated for three broad-leaved tree species red maple (*Acer rubrum*), white birch (*Betula papyrifera*), and red oak (*Quercus rubra*) in a two-year lysimeter experiment, with metal contamination and drought as treatments in a factorial design. Ni and Cu were the main elements of concern, but also Co and Zn had elevated levels in the contaminated substrate. Tissue concentrations of the four metals responded differently to the experimental treatments. Results show that metals influence drought stress at leaf, plant and at the whole system level. Metals influence plant water relationships by reducing the rate of transpiration and decreasing leaf relative water content (RWC). However, substrate moisture levels remain higher in metal-contaminated pots due to less transpiration from trees. Drought increased tissue concentrations of Cu. There was no significant interaction between the effects of metals and drought, indicating that their effects are additive. However, the strong response of traits such as transpiration rate to the combined stresses on certain days may be an indication that under extreme conditions metals can increase the drought effect to a fatal level.

Key words: heavy metals, drought, RWC, lysimeter, transpiration, trees,

2.2 Introduction:

Heavy metal toxicity often inhibits plant growth and ecosystem functions in regions affected by mining and smelting activities (Kozlov *et al*., 2009). Additionally, loss of nutrients and organic matter as a consequence of erosion following the damage to vegetation make such regions prone to drought (Poschenrieder and Barcelo, 2004). Heavy metals have been shown to decrease overall plant growth (Prasad, 2004) and negatively influence morphological traits relevant to plant water relations such as causing stunted roots (Kahle, 1993; Ryser and Emerson, 2007), and reduced size and even cause blockage of xylem vessels (Lamoreaux and Chaney, 1977; de Silva *et al*., 2012). Drought symptoms in metal experiments are a reoccurring theme in literature (Prasad, 2004), but studies with combined metal and drought stresses are only few and have so far resulted in contradictory findings. Plant responses to combined stresses are not always as predictable as in studies on individual stressors (Poschenrieder and Barcelo, 2004). Metal stress can add to drought stress by triggering a further drought response, such as in *Acer rubrum* (de Silva *et al.,* 2012), but plant responses to metals can also alleviate the effects of drought, as described for *Quercus suber* (Disante *et al*., 2011; 2014). In *Betula papyrifera*, exposure to metals reduced the plant's ability to respond to improved water availability, and actually increasing water availability in the substrate (Santala and Ryser, 2009).

Landscapes that have been contaminated with heavy metals share similar properties, with sparse vegetation, little organic cover, and severe erosion (Feisthauer *et al*., 2006; Kozlov, 2005; James and Courtin, 1985; Amiro and Courtin, 1981). Restoration efforts to reclaim such areas have identified common problems hampering recovery, such as low soil pH and metal toxicity, and as a further growth-limiting factor, drought (Amiro and Courtin,1981; James and Courtin, 1985; Wong, 2003; Kozlov,

2005). This is probably going to be an increasing problem with climate change increasing drought events (IPCC, 2007). Sudbury, Ontario is a region known for over a century of atmospheric metal disposition from Cu-Ni smelting processes (Winterhalder, 1996). Soils are known to be metal contaminated, and the area has been and still is part of an extensive vegetation re-greening program starting over 35 years ago (Gunn, 1995; SARA Group, 2009; Monet, 2012). However, establishment of new plants can still be hampered by drought, especially in areas that have low tree canopy cover (Santala, 2014). Therefore, it is important to understand the constraints metal-contaminated soils pose on plant drought tolerance, in order to ensure the success of large-scale remediation projects to rehabilitate forests in metal impacted landscapes. Published literature indicates, that to fully understand the simultaneous effects of metal and drought for plant-water relationships, studies needs to include leaf and whole plant levels to understand ecosystem level process.

The purpose of this study was to investigate the influence of soil heavy metal contamination, similar to that found in the Sudbury region, on plant water relationships in juvenile deciduous tree stands, focusing on the effects of periods of drought. The goal was to understand relationships between organ level, plant level and ecosystem level responses. The few existing investigations of plant-water relations on metal-contaminated substrate have shown the complexity of the issue, because close-to-natural situations with diverse vegetation and heterogeneous soil are difficult to recreate (Menon *et al*., 2005; Hermle *et al.* 2006). The goal of the present experiment was to gain understanding of plant water relations in juvenile deciduous tree stands on contaminated and non-contaminated soils at the different organizational levels, from individual leaves to the system as a whole, when plants were grown on a homogenous substrate mixture in

lysimeters with a controlled amount of water, with drought and non-drought treatments over two years.

Based on existing studies, I hypothesize that plant water use will decrease in response to exposure to heavy metals due to reduced root systems (Menon *et al*., 2005; Disante *et al*., 2010), impaired hydraulic conductivity (de Silva *et al*., 2012) and decreased transpiration (Pandey *et al*., 2002; Disante *et al*., 2011). This could result in water reserves lasting longer, possibly counteracting the effects of drought to some extent. Three species were used in this investigation, because different species may respond in a different manner. As diurnal and seasonal variation in temperature, with occasional extreme values, is also likely to contribute to the investigated stress effects, the experiment was conducted under close-to natural conditions outdoors, but covered with a plastic canopy to be able to regulate the water supply. As the duration of exposure probably plays an important role, the experiment was conducted over two growing seasons.

2.3 Materials and Methods:

Study species:

Three tree species, all occurring naturally in the study area, were used for the investigations. *Acer rubrum* and *Quercus rubra* can be classified as intermediate to late successional, and *Betula papyrifera* is an early successional shade-intolerant species (Kubiske and Pregitzer, 1996). Drought tolerance of the species increases in order of *B. papyrifera, A. rubrum* and *Q. rubra* (Turnbull *et al*., 2002, Daley and Phillips, 2006). Stunted individuals of *Q. rubra* often occur on dry rocky ridges towards the northern limit of its range (Farrar, 2007). In more southern regions, *A. rubrum* can occur in swamps (Farrar, 2007). All species survived the smelter-caused acid precipitation and

metal contamination in the Sudbury region during the 20th century as stunted multi-stemmed individuals, and can occur on dry rocky sites (Amiro and Courtin 1981). In dry summers, *B. papyrifera* may show drought-induced senescence and early leaf abscission (Pers. Observation; James and Courtin, 1987).

Experimental set-up:

Lysimeters were established in April, 2011 in Val Caron, Ontario, Canada (46°37'46.26" N 81°03'00.37"W) and maintained until September 2012 under a 6.4 m x 14.6 m plastic greenhouse with both ends of the greenhouse open for air exchange. The plastic cover resulted indoors in a temperature about 5°C higher than outdoors, on average. One hundred and seventy six pots (61 cm x 41 cm x 23 cm) were set up on 20 cm high wooden stands, and subjected to 4 experimental treatment combinations in a factorial design, with two watering regimes and two levels of substrate heavy metal contamination with 12 replicates each for each of the 3 investigated tree species. Additionally, eight pots of each treatment were left without trees to monitor evaporation from the pots without transpiration. To simulate canopy-shading effects, artificial saplings made of wood and plastic were placed in these pots. The lysimeters were arranged in 4 double rows, oak and maple in the middle of the rows in groups of four (two metal treatments × 2 species) alternating with drought and no-drought treatments. To avoid shading by the larger trees, birch lysimeters were placed at both ends of the rows in groups of four with pot of each treatment in a group. Lysimeters with no trees were arranged in blocks of eight, one block in each double row.

Each pot had a 1.3 cm wide drainage hole with a 1 L collection bottle beneath. The hole was covered with a scouring pad to prevent substrate loss. In each pot 40 L of

sieved substrate was filled to a depth of 16 cm, topped with 5 cm crushed white quartz (pH 6.7) to minimize evaporation (Appendix 2.2). The substrate used was a silt loam with pH of 5.0, consisting of 47% fine to coarse sand (50 µm-2 mm), 53% silt (<50 µm), and 0% clay with a specific surface area of 174 mm^2 mm^{-3} (Whyte and Son Aggregates, Dowling, Ontario). In the control treatment the substrate was uncontaminated, in the metal-contaminated treatment it was mixed with 5% v/v crushed 2 mm-sieved Cu-Ni slag with 6% of particles <50 µm and a calculated specific surface area of 29 mm^2 mm^{-3} (Fisher-Wavy and Vale, Sudbury, ON). Particle size analyses were conducted by Geoscience Laboratories (Sudbury, Ontario). The substrates were passed through a 1 cm sieve to remove stones and twigs and homogenized with a cement mixer for 15 minutes per 40 L pot. Substrate was chosen based on preliminary tests to have pH of 5.0, similar to that of the Sudbury Soils (Santala, 2014).

Phyto-available elements of the growth substrates were analyzed after a weak acid (1 M ammonium acetate) extraction process from samples collected at the end of the experiment, and the total elemental contents of the leachate was analyzed from samples collected at the end of each drought period. All analyses were conducted using plasma atomic emission spectroscopy (Testmark Inc., Sudbury, Ontario, Canada). There was a significant increase of Co, Cu, Ni and Zn in response to the inclusion of the crushed slag (Tables 2.1, 2.2). Slag-contamination of the substrate also increased concentrations of all four above-mentioned metals in the leachate (Tables 2.3; 2.4).

At transplantation, all of the pots were watered to field capacity (35% v/v moisture content). The moisture was kept approximately at that level by adding 1 liter of well water each day. For half of all of the lysimeters (with and without trees), three drought periods were applied during the two experimental seasons by not adding water

until soil water content reached values of 5-10% (pots without trees did not reach this level). In 2011 the period without water lasted from June 13 to July 18 when the pots were re-watered to field capacity, and from July 18 to August 25, after which field capacity was maintained. In 2012, pots in the drought treatment were not watered from 6 July until August 23. For birch the first drought period was delayed by two weeks as the seedlings were slow in recovering from transplantation. At the end of the first drought period, that coincided with a period of hot weather, maple and oak trees lost a large part of their leaves due to desiccation in 3 control pots. The trees recovered, and to avoid this to re-occur at the later stages of the other drought periods, substrate moisture in the control pots with the highest transpiration rates was prevented from going below 5-10% by additional watering until all drought treatments of most pots had dried to about 10% soil moisture. The drought treatment did not have a significant effect on substrate metal availability (Tables 2.1; 2.2).

Trees were planted in 144 of the pots in May 2011. In each of these pots six saplings of either red maple (*Acer rubrum*), red oak (*Quercus rubra*) or white birch (*Betula papyrifera*) were planted. Maple and oak saplings were commercially obtained (Botanix Azilda Greenhouses, Azilda, ON). The seed stock originated from Tree Seed Zone 37 in Southern Ontario. At planting, the freshly pruned saplings were 2-years old and their height ranged between 30-100 cm with no branches and no fine roots. Birch seedlings, 2 cm in height, were collected in June 2011 in Sudbury on uncontaminated ground, and transplanted into lysimeters. Roots were thoroughly washed prior to transplanting to remove any organic matter. At the beginning of each experimental season, 600 mL of nutrient solution was added to each pot. The nutrient solution was prepared using Home Gardener All Purpose 20-20-20 fertilizer (St. Jacobs, Ontario) and

additional $MgSO_4$ and $CaCl_2$. The solution contained 4.7 mM N, 0.92 mM P, 1.4 mM K, 4.3 mM, 4.3mM CaCl, 2.2 mM Mg, 2.2 mM SO_4, 6.1 µM B, 2.6 µM Cu, 5.9 µM Fe, 3.0 µM Mn, 2.5 µM Zn , 0.02 µM Mo, and 14 µM EDTA.

From November 2011 to April 2012 the plastic cover of the greenhouse was removed, the plants placed directly on the ground, and surrounded with bales of straw and covered with freshly chipped wood chips and loose straw for insulation during the winter months. After snowmelt in mid April 2012 the pots were placed back onto the stands and a new plastic was mounted on the greenhouse for a second season of the experiment.

Lysimeter measurements:

Soil moisture at the top 5 cm was measured weekly during each drought period on six locations per pot, using HH2 Moisture Meter and SM200 Moisture sensor (Delta-T Devices, Cambridge, UK) (Table 2.5). Moisture at the bottom 5 cm was measured at the end of each drought period with a PR2 profile probe in a 50 cm tube (Delta–T Devices, Cambridge, UK). (July 2011, August 2011, August 2012). The relationship between moisture content and water potential in the experimental substrates was assessed in both substrates by periodically measuring the moisture content with the Soil Moisture Sensor (SM200)and the water potential with an equitensiometer, (EQ2-UM-1.3, Delta-T Devices, Cambridge, UK) in a drying pot of each substrate type. Slag in the substrate did not influence the relation between substrate moisture readings and water potential. Soil water contents of 15%, 10% and 5% corresponded to -50, -100 and -150 kPa water potential, respectively. However, it is important to note that near the end of the drought

periods the tensiometer connection with the substrate may have broken down due to the large difference in pore volume in the dry sandy substrate (Morgan *et al*., 2001).

To measure the total water loss from the lysimeters, all drought treatment pots were weighed three times over the course of each drought period. A Moultrie digital game scale (MFH-DS, 330 lbs, USA) was attached to a powered winch on a wooden frame that could be moved around in the greenhouse (Fig. 2.1).

Plant Measurements:

Canopy Leaf Area Index (LAI):

LAI of the canopies in the lysimeters was measured at the end of both drought periods in 2011 using a LI-COR LAI-2000. Canopy cover was calculated based on 15 readings per lysimeter, 12 below the canopy and 3 above the canopy to compensate for variation in ambient light due to changes in cloud cover or irregularities in the greenhouse plastic. All readings were conducted on overcast days. Four readings per pot were conducted for both red maple and red oak. White birch canopy height was not sufficiently high for proper readings.

Relative Water Content (RWC) and other leaf traits:

Leaves were collected at the end of each drought period, with exception of birch at the first harvest due to its small size. To measure leaf relative water content three leaves per pot from three different saplings were collected between 14:00-15:00 in the afternoon. The leaves placed in individual pre-weighed labeled plastic bags following the protocol described in Tanentzap *et al.* (see Chapter 5), and weighed with the bag in the

laboratory within 24 hours. The leaves were then placed between moist paper towels for over 12 h to measure their saturated mass, following the protocol described in Ryser *et al.* (2008). Dry mass of all leaves was measured after drying at 75°C for 48 hours. Relative water content was calculated according to Whiting *et al.* (2003):

RWC = [(fresh mass-dry mass)/(saturated mass-dry mass)] (1) .

The leaves collected for the measurement of relative water content were also used for other measurements. After the leaves had reached full turgor their chlorophyll content (SPAD-502, Konica Minolta Sensing, Inc. Japan), lamina thickness (Mitutoyo Deep Throat digital micrometer caliper No-547-520), and leaf area (LI-3100, LI-COR Inc Nebraska, USA) were recorded. All measurements were repeated twice and the averages were used for the analyses. Specific leaf area (SLA) was calculated as leaf area per leaf dry mass, and leaf dry matter content (DMC) as leaf fresh mass to dry mass ratio.

To measure stomatal density, three leaves per pot from three different saplings were harvested after the second drought period in the first year. Clear nail varnish was applied in a 1 cm^2 section of the leaf on a similar place as used for leaf chlorophyll measurements. Dried nail varnish was then peeled and placed on a microscopic slide. Microscopic images were taken from 5 locations of each leaf peel at random using ×100 magnification for *Acer rubrum* and *Betula papyrifera* and × 40 magnification for *Quercus rubra*.

Five further replicate leaf samples for each species and treatment were collected after the August 2011 and August 2012 drought periods. The leaves were analysed for their total metal content (Testmark Inc., Sudbury, Ontario, Canada) using inductively coupled plasma-mass spectrometry method (Method 6020A).

Stomatal resistance and lamina temperature:

Stomatal resistance was measured at the end of both drought periods, using a porometer (LI-Cor Model LI-60, SN: R488-7504), calibrated at 27°C and 32 °C. Three leaves per pot from three different trees were measured. All leaf measurements were taken at peak sun hours on cloudless high temperature days between 1:00 and 3:00 pm. Time and temperature were recorded for each measurement and repeated for 3 leaves per pot for 6 treatment replicates.

Leaf lamina temperature was measured using an infrared thermometer (Fisher Scientific 15-077-968) on leaves of similar age, angle, orientation, and height within the canopy on cloudless high temperature days between 1:00 and 3:00 pm.

Sapling Dry Mass measurements:

All stems were harvested at the end of the two-year experiment. Stems were cut at soil surface and placed in large paper bags to let air dry for 2 months. Dry mass was measured after drying at 75°C for one week. Root dry mass was determined by collecting at the end of each year, six 5-cm cores from 3 replicate pots per treatment and species . Roots were thoroughly washed to remove any substrate. Dry masses of the root samples were measured after drying at 75°C for 48 hours.

Statistical Analysis:

The responses to the experimental treatments of all plant growth and functional traits that were measured more than once on the same pot were tested using linear mixed effects models. Metal treatment, drought treatment, and harvest date were specified as predictors, along with all their two-way interactions and the three-way interaction among

these variables. Species was also included as a fixed factor predictor, with pot identity specified as a random effect to account for repeated measures. Models were fitted with restricted maximum likelihood and the significance of terms was tested using log-likelihood ratio tests. All factors and combinations of interactions that were non-significant ($p > 0.05$) were not reported in the final model. The same procedure was used for water loss over time and soil moisture, and a linear model without the random effect was used to test the responses of stomatal density and stem dry mass as these were measured only once per pot. I used the same linear model to compare among treatments the concentration of Co, Cu, Ni, Zn in the soil substrate, leachate, and in leaves pooled from three individuals per pot. Leaf DMC and leaf area were square root transformed, and all other traits aside from temperature and RWC were log-transformed prior to fitting models to ensure normality of residuals. All analyses were carried out using R 3.0.2 (R Foundation for Statistical Computing Platform, 2013).

2.4 Results:

Plant Growth:

Both applied stresses – heavy metals and drought – decreased plant growth. Heavy metals decreased average leaf mass and average leaf area by 15-24% for the three studied species, with the effects increasing over time (Fig. 2.2, Table 2.7). The reduction in these variables at the last harvest was 37-54%. There also was a decrease in LAI due to drought, and in the second LAI measurement also due to metals. The results of the first LAI measurement were not conclusive due to drought-induced leaf abscission in some of the control pots, as the large plants consumed over a short period more water than was supplied. Metals reduced stem dry mass in birch, the species that had grown on

the contaminated substrate starting as a small seedling, whereas for maple and oak with a considerable stem mass at planting the metal effect was not significant. Drought reduced root growth in maple and in oak. Drought reduced stem mass of all species, but the effects were not significant. Metals reduced root dry mass of maple by close to 40%, but for the other species there was no significant effect on root dry mass. Overall, there was no significant metal × drought interaction for any of the measured traits indicating that metal and drought stresses affected plant growth in an additive manner (Table 2.7).

The measured plant traits SLA, leaf dry matter content and leaf thickness were not significantly affected by the applied stresses (Appendix 2.1a,b). Metals decreased leaf chlorophyll content of oak and birch, but not of maple. Drought increased the chlorophyll content in all species (Appendix 2.1a,b).

Plant water relationships:

Overall, the measured variables related to plant-water relationship were influenced both by the metal and drought treatments. Stomatal resistance increased both in the metal and the drought treatments both in *A. rubrum* and *Q. rubra,* with the strongest effect in oak (Fig. 2.3, Table 2.8). Leaf RWC was significantly reduced by metals in maple and by drought for all three species (Fig. 2.3, Table 2.8). Leaf temperature was increased both by metal and drought treatments, however the effects were not significant due to the large variation. This variation in treatment effects on leaf temperature seemed to be depending on environmental temperature because the treatments had an effect on leaf temperature mainly on hot sunny days (Fig 2.3). In a General Linear Model including RWC as co-variable, RWC was significantly correlated

with leaf temperature (Table 2.8). Neither metals nor drought had an effect on stomatal count.

Effects on substrate and ecosystem-level:

There was a significantly higher concentration of plant-available Co, Cu, Ni and Zn in the metal-treated substrate compared to the pure sand (Tables 2.1, 2.2). Neither species, nor the presence of trees in general, had any significant effect on the substrate metal availability (Tables 2.1, 2.2). Drought treatment did not have a significant effect on the substrate metal content (Tables 2.1, 2.2), but decreased substrate pH (Table 2.10). There was a significant increase of metals (Co, Cu, Ni and Zn) in the leachate due to the metal treatment (Tables 2.3, 2.4), and the drought treatment did not have an effect on the amount of metals in the leachate. The metal treatment resulted in a higher leaf tissue concentration of Ni and Co and the drought treatment in higher concentrations of Ni and Cu in the leaves (Fig. 2.4, Table 2.9). In birch, metal treatment reduced leaf Zn concentrations (Fig 2.4).

Substrate moisture content was significantly higher in the metal-contaminated pots than in pots with no metals (Tables 2.5, 2.6). However, this was the case only for pots with plants, indicating that this is a transpiration, not an evaporation effect. The increased moisture in metal-contaminated pots matched the water loss data, which showed that pots with metal-contaminated substrate lost less water over time than uncontaminated pots, but only in case plants were present (Tables 2.11, 2.12).

Table 2.1. Co, Cu, Ni and Zn bioavailable concentrations (ppm) using 1 M ammonium acetate for extraction in substrate of pots within four different treatments (control: no metal, no drought, drought only, metal only, and metal and drought) in factorial combinations (± 1 S.E.).

	Control	**Drought**	**Metal**	**M+D**
Co	0.024 ± 0.006	0.025 ± 0.006	0.233 ± 0.053	0.314 ± 0.072
Cu	0.077 ± 0.018	0.057 ± 0.013	6.505 ± 1.492	6.301 ± 1.446
Ni	0.063 ± 0.014	0.063 ± 0.014	3.048 ± 0.699	4.051 ± 0.929
Zn	0.197 ± 0.045	0.121 ± 0.028	0.236 ± 0.054	0.279 ± 0.064

Table 2.2. Fitted linear model, fitting the model using test statistics for bioavailable soil metal analysis (Co, Cu, Ni and Zn; ppm) in substrate of pots within four different treatments (control: no metal, no drought, drought only, metal only, and metal and drought) in factorial combinations (± 1 S.E.).

Metal	Treatments	t_{71}	p-value
Co			
	Intercept	-27.94	**<0.001**
	metal	22.81	**<0.001**
	drought	-0.06	0.9562
	species	-2.70	**0.008**
	metal × drought	0.07	0.948
Cu			
	Intercept	-6.42	**<0.001**
	metal	29.44	**<0.001**
	drought	-0.18	0.856
	species	-0.528	0.599
	metal × drought	-1.36	0.178
Ni			
	Intercept	-10.31	**<0.001**
	metal	33.68	**<0.001**
	drought	0.41	0.682
	species	-0.73	0.469
	metal × drought	-1.36	0.178
Zn			
	Intercept	-9.711	**<0.001**
	metal	11.28	**<0.001**
	drought	-0.16	0.872
	species	-0.83	0.41
	metal × drought	-0.52	0.607

Table 2.3. Total concentrations of Co, Cu, Ni and Zn (ppb; mean values ± 1 S.E.) in leachate from lysimeter pots with the three tree species (*Acer rubrum, Quercus rubra, Betula papyrifera*) and pots with no trees in four different treatments (control: no metal, no drought, drought only, metal only, and metal and drought) in factorial combinations.

A. rubrum	**Control**		**Drought**		**Metal**		**M+D**	
Co	15.69	±8.6	33.65	±20.2	208.89	±74.5	167.50	±66.5
Cu	61.10	±47.3	64.00	±8.7	231.10	±99	233.00	±36
Ni	78.52	±28.6	1024.00	±136	2168.17	±670	3420.00	±1720
Zn	41.45	±16.2	98.55	±2.4	221.60	±73.4	298.50	±190.5

Q. rubra	**Control**		**Drought**		**Metal**		**M+D**	
Co	5.46	±3.34	4.29	±2.29	148.85	±73.2	163.50	±26.5
Cu	11.22	±3.21	9.35	±2.45	248.12	±141	229.00	±88
Ni	60.28	±26.1	75.85	±14.9	1704.85	±709	3135.00	±155
Zn	27.98	±11.7	33.05	±0.35	168.43	±75.1	368.00	±12

B. papyrifera	**Control**		**Drought**		**Metal**		**M+D**	
Co	1.29	±0.68	4.28		87.11	±53.63	355.00	
Cu	14.85	±4.81	17.00		122.86	±83.7	598.00	
Ni	29.50	±8.17	128.00		906.40	±635	3620.00	
Zn	6.50	±1.56	46.30		98.70	±68.4	372.00	

No Trees	**Control**		**Drought**		**Metal**		**M+D**	
Co	15.94	±9.35	8.05	±2.45	174.20	±84.03	172.80	±87.2
Cu	23.62	±9.26	14.95	±2.65	206.68	±110	263.00	±113
Ni	95.92	±56.1	69.65	±15.1	1146.60	±435	2545.00	±1005
Zn	28.30	±8.87	39.20	±10	136.30	±46.9	325.00	±44

Table 2.4. Fitted linear models, fitting the models using test statistics for total leachate metal concentrations (Co, Cu, Ni and Zn) in substrate of pots within four different treatments (control: no metal, no drought, drought only, metal only, and metal and drought) in factorial combinations.

Metal	**Treatments**	**t_{58}**	**p-value**
Co			
	Intercept	0.58	0.567
	metal	7.49	**<0.001**
	drought	0.33	0.746
Cu	Intercept	2.85	**0.006**
	metal	7.30	**<0.001**
	drought	0.39	0.698
Ni	Intercept	3.80	**<0.001**
	metal	9.10	**<0.001**
	drought	1.30	0.202
Zn	Intercept	3.99	**<0.001**
	metal	5.95	**<0.001**
	drought	0.31	0.76

Table 2.5. Substrate moisture (% v/v) in lysimeter pots at the end of each drought period with the three tree species (*Acer rubrum, Quercus rubra, Betula papyrifera,* no trees) in four different treatments (control: no metal, no drought, drought only, metal only, and metal and drought) in factorial combinations over 3 drought periods (June:July-2011, July:Aug-2011, July:Aug-2012; Mean values ± 1 S.E.).

A. rubrum	**Control**	**Drought**	**Metal**	**M+D**
July-2011	29.09 ± 1.47	14.80 ± 1.16	30.07 ±1.86	16.92 ±0.85
Aug.-2011	22.90 ± 2.03	4.20 ± 0.90	25.85 ±2.19	12.23 ±1.34
Aug.-2012	19.62 ± 2.50	4.38 ± 1.19	21.58 ±2.74	6.18 ±1.62

Q. rubra	**Control**	**Drought**	**Metal**	**M+D**
July-2011	28.98 ± 1.45	3.01 ± 1.36	30.92 ±1.31	8.59 ±2.13
Aug.-2011	21.17 ± 2.42	3.02 ± 1.40	26.00 ±1.79	3.48 ±0.69
Aug.-2012	20.28 ± 1.86	11.94 ± 3.42	25.20 ±1.70	7.96 ±2.30

B. papyrifera	**Control**	**Drought**	**Metal**	**M+D**
Aug.-2011	22.97 ± 1.57	10.53 ± 2.82	28.78 ±1.94	9.47 ±1.44
Aug.-2012	22.13 ± 2.34	5.62 ± 2.11	24.43 ±2.37	8.85 ±2.61

No Trees	**Control**	**Drought**	**Metal**	**M+D**
July-2011	34.70 ± 1.77	25.83 ±3.57	36.73 ±2.25	29.15 ±2.63
Aug.-2011	34.55 ± 2.55	21.88 ±1.39	35.90 ±2.86	20.63 ±1.44
Aug.-2012	32.85 ± 3.18	21.63 ±5.55	32.93 ±2.38	16.95 ±1.64

Table 2.6. Log-likelihood ratio tests comparing substrate moisture (% v/v) in lysimeter pots among three tree species (*Acer rubrum, Quercus rubra, Betula papyrifera)* over time 4 times within each of the 3 drought periods (Jul-2011, Aug-2011, Aug-2012). Trees were exposed to four different treatments: control (no metal, no drought), drought only, metal only, and metal and drought. χ^2 = chi-square statistic; df = degrees of freedom**.**

Treatments	χ^2	**df**	**p-value**
metal	8.096	1	**0.004**
drought	53.82	1	**<0.001**
species	127.2	4	**<0.001**
metal × drought	8.097	2	**0.017**
drought × time	18.18	3	**<0.001**
metal × time	12.58	3	**0.006**
metal × day	55.96	14	**<0.001**

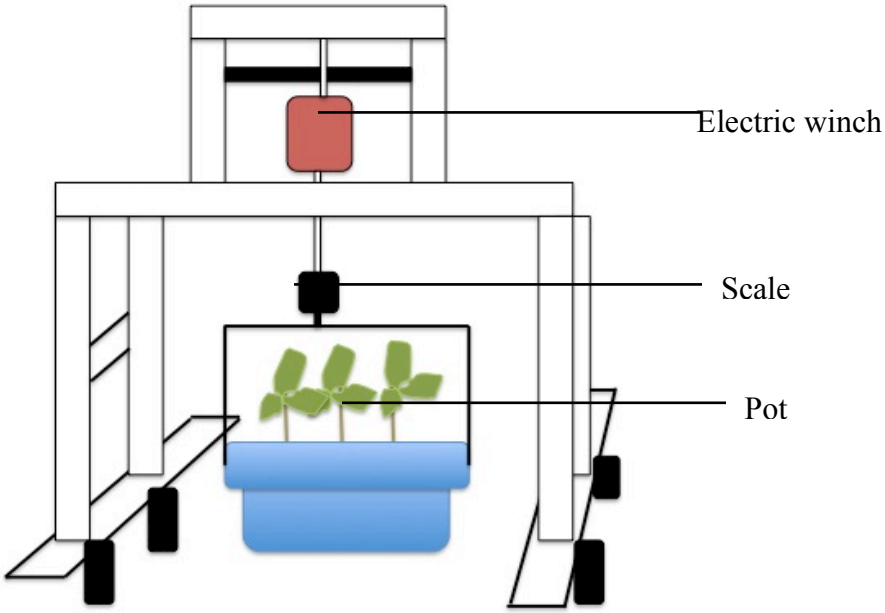

Fig. 2.1. Schematic diagram of the apparatus for lifting and weighing individual pots. An electric winch was attached to a digital scale that was able to lift and weigh each drought pot over the experimental period.

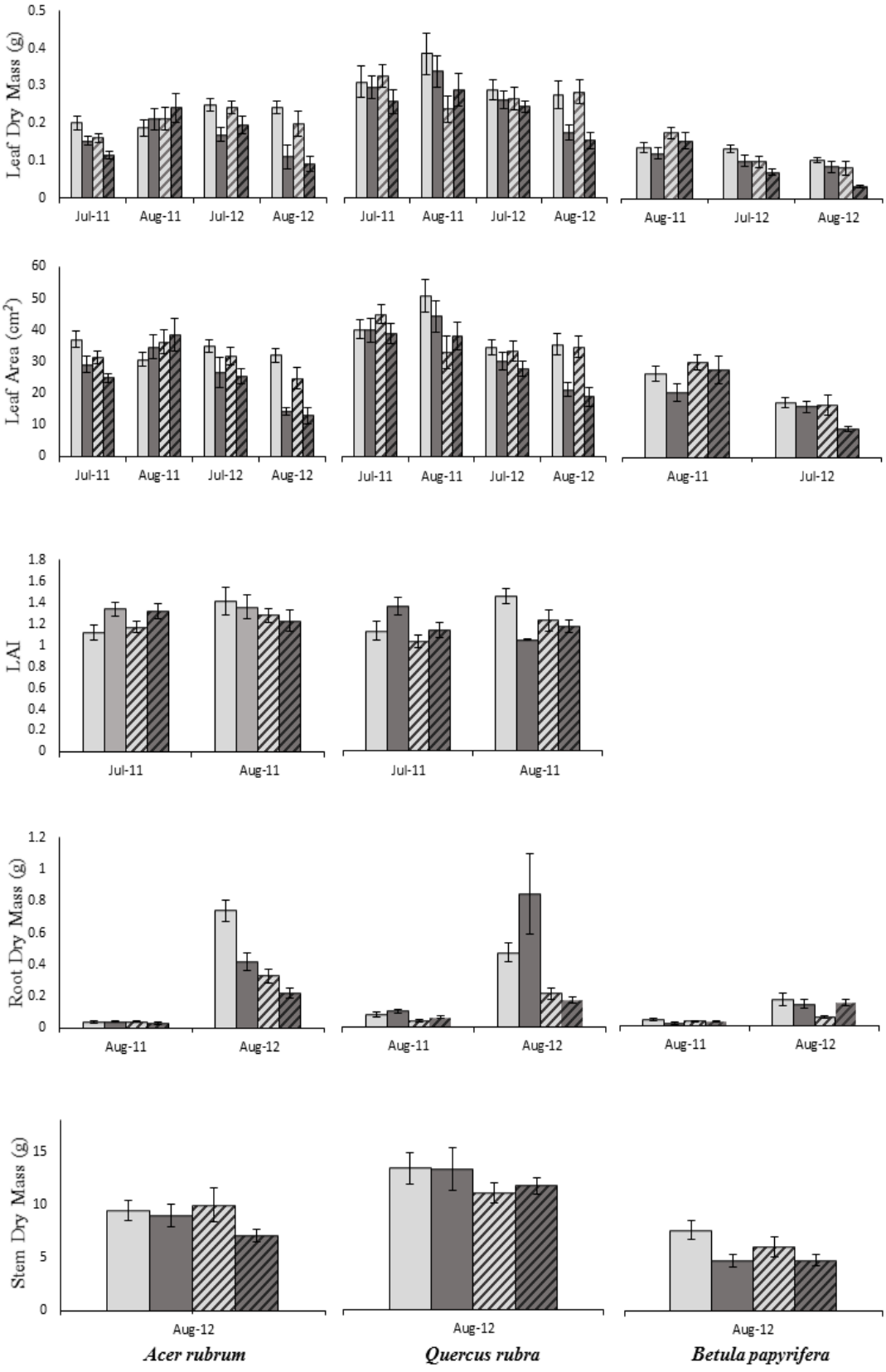
Leaf Dry Mass (g)
0.5
0.4
0.3
0.2
0.1
0
Jul-11
Aug-11
Jul-12
Aug-12
Leaf Area (cm²)
60
50
40
30
20
10
0
LAI
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Root Dry Mass (g)
1.2
1
0.8
0.6
0.4
0.2
0
Stem Dry Mass (g)
15
10
5
0
Acer rubrum
Quercus rubra
Betula papyrifera

Fig. 2.2. Mean values of plant growth traits (Error bars: ± 1 S.E.) among three species. Traits were: leaf dry mass (g), leaf area (cm^2), LAI, root dry mass (g), and stem dry mass (g) in *Acer rubrum, Quercus rubra, Betula papyrifera.* The variables were measured in July and August of 2011 and 2012, except for root dry mass in August 2011 and 2021, and stem dry mass in August 2012 only. Trees were exposed to four different treatments: control: (light fill); metal only: (dark fill); drought only: (light hatched fill); and metal and drought: (dark hatched fill).

Table 2.7. Log-likelihood ratio tests comparing plant growth traits among three tree species. Traits were: leaf dry mass, leaf area, LAI, root dry mass and stem dry mass measured in *Acer rubrum, Quercus rubra, Betula papyrifera* in both July and August of 2011 and 2012. Trees were exposed to four different treatments: control (no metal, no drought), drought only, metal only, and metal and drought. Interactions not included in table were none significant. χ^2 = chi-square statistic; df = degrees of freedom.

Traits	**Treatment**	**χ^2**	**df**	**p-value**
Leaf Dry Mass (g)				
	metal	41.31	1	**<0.001**
	drought	14.05	1	**<0.001**
	species	154.93	2	**<0.001**
	harvest	76.49	3	**<0.001**
Leaf Area (cm^2)				
	metal	37.66	1	**<0.001**
	drought	5.65	1	**0.017**
	species	117.11	2	**<0.001**
	harvest	105.36	3	**<0.001**
LAI				
	harvest	7.89	1	**0.005**
	metal × harvest	15.36	1	**<0.001**
Root Dry Mass (g)				
	drought	11.61	1	**<0.001**
	species	34.65	2	**<0.001**
	harvest	127.76	1	**<0.001**
	species× harvest	2.93	2	**0.004**
		t_{130}		**p-value**
Stem Dry Mass (g)				
	intercept	25.83		**<0.001**
	metal	-1.794		0.0752
	drought	-1.297		0.197
	species Betula	-4.979		**<0.001**
	species Quercus	4.734		**<0.001**
	metal × drought	0.237		0.813

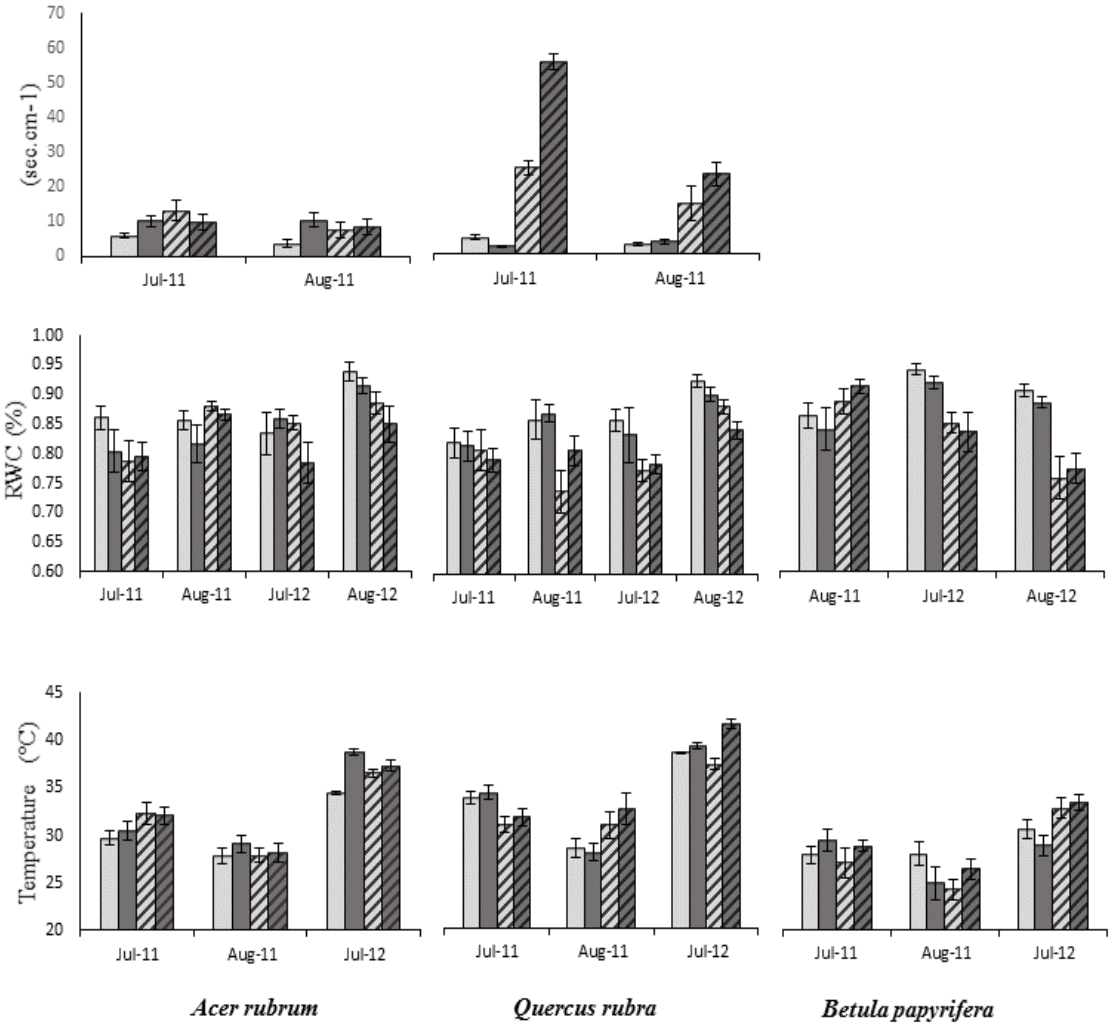

Fig. 2.3. Mean values of plant traits (Error bars: ± 1 S.E.) among three species. Traits were: stomatal resistance (sec. cm-1), RWC (%) and temperature (°C) measured in *Acer rubrum, Quercus rubra, Betula papyrifera* in both July and August of 2011 and 2012. Trees were exposed to four different treatments: control: (light fill); metal only: (dark fill); drought only: (light hatched fill); and metal and drought: (dark hatched fill).

Table 2.8. Log-likelihood ratio tests comparing foliar traits among three tree species. Traits were: stomatal resistance (sec. cm-1), RWC (%) and temperature (°C), measured in *Acer rubrum, Quercus rubra, Betula papyrifera* in both July and August of 2011 and 2012. Trees were exposed to four different treatments: control (no metal, no drought), drought only, metal only, and metal and drought. χ^2 = chi-square statistic; df = degrees of freedom.

Trait	Treatment	χ^2	df	p-value
Stomatal Resistance				
	drought	22.23	1	**<0.001**
	harvest	10.40	1	**<0.001**
	metal × harvest	6.56	1	**0.010**
	drought × harvest	4.622	1	**0.032**
RWC				
	metal	3.85	1	**0.049**
	drought	34.37	1	**<0.001**
	species	9.55	2	**0.008**
	harvest	32.38	3	**<0.001**
	drought × harvest	16.55	3	**<0.001**
Temperature (°C)				
	species	41.01	2	**<0.001**
	harvest	141.88	2	**<0.001**
	drought × harvest	7.54	2	**0.023**

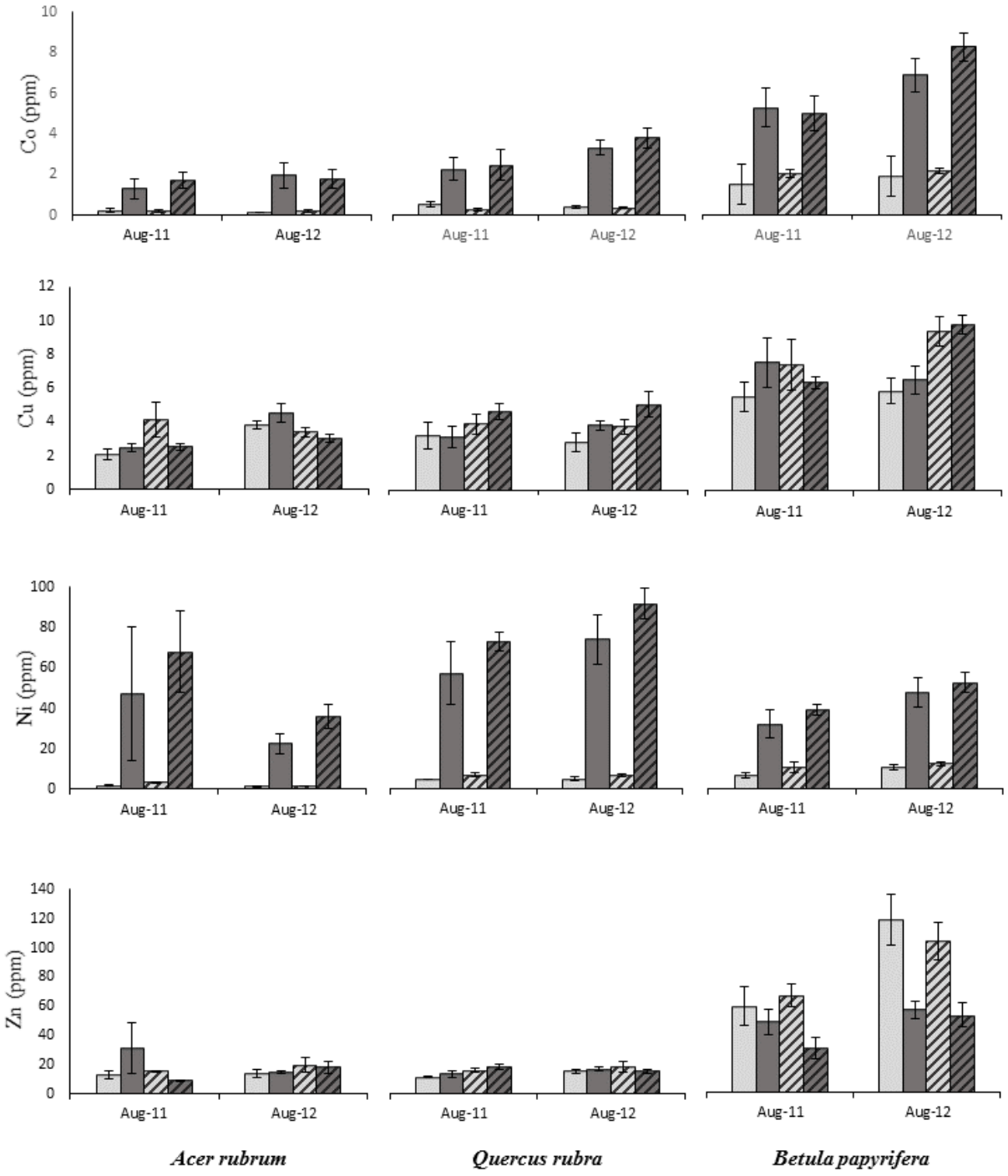

Fig. 2.4. Total metal concentrations (Co, Cu, Ni and Zn (ppm); Error bars: ± 1 S.E.) in leaves of three deciduous species (*Quercus rubra, Acer rubrum, Betula papyrifera*) in four different treatments (control: (light fill); metal only: (dark fill); drought only: (light hatched fill); and metal and drought: (dark hatched fill)) collected at the end of each drought year in Aug.-2011 and Aug.-2012.

Table 2.9. Fitted linear model, fitting the model using test statistics for metal concentrations (Co, Cu, Ni and Zn (ppm)) in leaves of pots within four different treatments (control: no metal, no drought, drought only, metal only, and metal and drought) in factorial combinations collected at the end of each drought year.

Metal	Treatments	t_{110}	p-value
Co			
	Intercept	-6.47	**<0.001**
	metal	7.98	**<0.001**
	drought	0.43	0.666
	time	0.35	0.729
	species *Acer*	-5.22	**<0.001**
	species *Quercus*	9.76	**<0.001**
	metal × drought	0.26	0.798
Cu			
	Intercept	9.29	**<0.001**
	metal	1.12	0.264
	drought	2.88	**0.004**
	time	1.51	0.133
	species *Acer*	-1.61	0.109
	species *Quercus*	8.58	**<0.001**
	metal × drought	-1.22	0.225
Ni			
	Intercept	0.441	0.66
	metal	4.883	**<0.001**
	drought	0.077	0.938
	time	0.14	0.889
	species *Acer*	-2.48	**0.015**
	species *Quercus*	8.89	**<0.001**
	metal × drought	0.11	0.9151
Zn			
	Intercept	19.11	**<0.001**
	metal	1.26	0.211
	drought	1.76	0.0809
	time	2.44	**0.0162**
	species *Acer*	0.631	0.529
	species *Quercus*	14.08	**<0.001**
	metal × drought	-2.61	**0.01**

Table 2.10. Substrate pH (± 1 S.E., n=6) values in in four different treatments (control: no metal, no drought, drought only, metal only, and metal and drought) collected at the end of each drought period (July 2011, Aug. 2011 and Aug. 2012).

	Control		**Drought**		**Metal**		**M+D**	
July 2011	4.80	±0.28	4.25	±0.10	4.51	±0.16	4.56	±0.12
Aug. 2011	4.82	±0.20	4.28	±0.12	4.98	±0.19	4.52	±0.05
Aug. 2012	4.84	±0.06	4.48	±0.05	4.69	±0.07	4.53	±0.07

Table 2.11. Water loss from lysimeters (kg ; ± 1 S.E.) for three tree species (*Acer rubrum, Quercus rubra, Betula papyrifera*) and pots with no trees during the three drought periods (July-2011, Aug,-2011 and Aug.-2012) in two treatments (drought only; metal + drought).

	Treatment	**July-11**		**Aug.-11**		**July-12**	
No Trees	Drought	1.38	±0.29	2.30	±0.55	2.26	±0.65
	M+D	1.23	±0.91	2.21	±0.31	3.30	±0.90
A. rubrum	Drought	2.53	±0.55	5.62	±1.22	5.52	±1.32
	M+D	2.51	±0.41	3.57	±0.52	3.59	±0.69
Q. rubra	Drought	3.87	±1.09	3.69	±0.92	3.43	±0.54
	M+D	2.68	±0.84	3.13	±0.64	3.62	±0.72
B. papyrifera	Drought			4.54	±0.57	3.57	±0.46
	M+D			3.56	±0.31	3.14	±0.44

Table 2.12. Log-likelihood tests for water loss in three tree species (*Acer rubrum, Quercus rubra, Betula papyrifera*) and pots with no trees during the three drought periods (July-2011, Aug,-2011 and Aug.-2012) in two treatments (drought only; metal + drought). Interactions not included in table were none significant.

Treatment	χ^2	**df**	**p-value**
Metal	45.15	1	**<0.001**
Species	7.86	2	**0.020**

2.5 Discussion:

The results demonstrate that heavy metal contamination of the substrate can have a strong effect on plants' drought responses, as contamination influences plant water relations at the levels of single leaves, individual plants, and at the level of the whole pot. Metals reduced the rate of transpiration and leaf relative water content (RWC) in all three investigated tree species. Despite these responses, which were similar to those triggered by drought, in metal-contaminated pots the available substrate moisture level was actually higher than in the uncontaminated pots, as a result of the reduced transpiration. The effects of the individual stressors, metal and drought, were mostly additive, such as in case of plant growth, but in some cases, such as transpiration rates and leaf temperature, there was some indication that the combination of both stresses could have a synergistic effect with detrimental consequences for the plant.

The most distinct effect of metal contamination on plant water relationships was the reduced water use by the trees. Several potential mechanisms leading to reduced water use have been described in literature. Heavy metals such as Cd can cause stomatal closure in excised leaves (Bazzaz *et al.,* 1974), possibly by inhibiting leaf aquaporin function (Devi *et al*., 2012). In the present experiment, Ni and Co were found to accumulate in the leaves. These elements have been shown to reduce transpiration, but not to the same extent as for example Hg and Cd have (Sheoran *et al.,* 1990; Pandey and Sharma, 2002). A direct effect of leaf metals on stomatal conductance and transpiration in the present experiment also seems unlikely because of the low leaf relative water content of plants grown on the contaminated substrate, indicating that stomatal closure would be a response to drought stress experienced by the plants (Lambers, 2008). However, the plants have slightly more water available to them and uptake and/or

transport are probably the water-use-limiting steps. Metals can impede water uptake by reducing root growth (Kahle *et al.,* 1993), and inhibit root aquaporin function (Przedpelska-Wasowicz and Wierzbicka, 2011). However, Ni is not known to exert a strong effect on root aquaporins, in contrast to Hg and Cd, (Pandey and Sharma, 2002; Chapter 3 of this thesis). The experiment described in Chapter 3 showed a strongly reduced transpiration of white birch grown on metal- contaminated substrate, mainly as a result of the reduced leaf area, which also was observed in the present experiment. Plants grown on contaminated substrate in this experiment had a reduced root mass, which may have contributed to a reduced ability to take up water, as high relative biomass allocation to roots is a trait associated with drought tolerance of a species (Markesteijn and Poorter, 2009). A reduced hydraulic conductance may contribute to low relative water content of leaves of plants on contaminated substrate. Santala and Ryser (2009) and de Silva *et al*. (2012) found Cu/Ni containing slag having a damaging effect on xylem of *Betula papyrifera* and *Acer rubrum*, respectively. Lamereaux and Chaney (1977) found similar effects of Cd on *Acer saccharinum*. These authors observed cellular debris in xylem of metal-affected plants, possibly a result of oxidative damage caused by reactive oxygen species (ROS), which have been observed to increase as a result of Cu contamination (Zhang *et al.,* 2008). Such damaged xylem may be the cause of an increased vulnerability of red maple saplings on low water potentials (Tanentzap and Ryser, 2015).

Leaf temperature of the drought treatment plants were higher compared to the control, depending on harvest. This effect was more predominant in *Acer rubrum* and *Quercus rubra*, compared to *Betula papyrifera.* There was some indication that under drought, metal exposure resulted in higher leaf temperatures, but the effect was not significant. An effect of heavy metals on leaf temperature has been reported by Thakur

and Singh (2012) showing that there was a 3-degree increase in soybean leaf temperature in response to Cd exposure in a 16 day hydroponic study. Thakur and Singh (2012) actually suggest that leaf temperature is a good indicator for heavy metal pollutants. However, Cd is known as a stronger inhibitor of root growth (Fiala *et al.,* 2013), and transpiration (Sheoran *et al*., 1993) than Ni and in the present experiment temperature increases were less clear.

It has been observed earlier that trees on metal-contaminated soils use less water, leading to a slower drying out of the soil (Menon *et al.,* 2005; Santala and Ryser, 2009). It has been suggested by Disante *et al*. (2014) that a reduced water use by metal-exposed plants would reduce the vulnerability to drought as soil remains moist for a longer time. Also, exposure to metals results in smaller xylem vessels, which are a characteristic of drought tolerance (de Silva *et al.,* 2012). However, in the present experiment, relative water content was lower in the leaves of the metal-exposed plants compared to the control plants, despite the higher amount of water in the soil indicating that the reduced water use is a problem, caused by disrupted water acquisition capacity, rather than a protective trait. This is in contrast to results by Disante *et al.* (2014) who found a less negative effect by drought on stomatal conductance and photosynthetic rate of *Quercus suber* in the presence of elevated Zn concentrations. These results seem to indicate that Zn has different effects on plant water relations than the main contaminants Ni and Cu in the present study which aggravated the drought effects. Also, Disante *et al.* (2014) found lower levels of Zn tissue concentrations under low water treatment compared to high water treatment, whereas in the present study drought increased tissue Cu concentrations in oak and birch. There was a weak also in the present study for Zn a reduced Zn tissue concentration at drought in birch. The lower overall Cu accumulation in the leaves

compared to Ni may be due to Cu accumulation elsewhere within the woody plant such as build up within the xylem walls (Yruela, 2005). Cu is known to accumulate in roots rather than leaves due to special proteins (Rauser and Curvetto, 1980; Shen-Lin and Wu, 1994)

Leaf metal analysis showed that tissue concentrations of various metals responded to the treatments differently in general. There was a higher concentration of Co and Ni, but not Cu within the metal-treated plants of all three tree species. Interestingly, in birch Zn concentrations were lower in the metal treatment compared to the controls, despite significantly elevated soil levels. Birch had comparatively high Zn concentrations even under non-contaminated soils. High accumulation rate of Zn in another fast-growing tree, *Populus tremuloides* has been shown by Vollenweider *et al*. (2011). In contrast to other metals, Zn was stored in this species as globoid deposits in several organelles (Vollenweider, 2011b), indicating that there may be special mechanisms to deal with that metal. Zn was not a main contaminant in our study, but interestingly, tissue concentrations of Zn were lower in birch exposed to Ni, Cu and Co. This may indicate that the other metals disturbed the uptake/transport/storage of Zn in that species. The special physiological status of Zn was also supported by the results of Hogan and Rauser (1979), who found that a clone of *Agrostis gigantea* from Sudbury area, tolerant to Ni, Cu and Co, was not tolerant to Zn.

Despite the clear growth effects and effects on plant water relationships, plant growth traits SLA, LDMC and leaf thickness did not respond to the applied stresses. Although stresses are known to influence these traits (Ryser and Lambers, 1995), they seem to be better suited to describe interspecific and ontogenetic differences, which were clearly higher than the stress-caused effects. The higher SLA, and lower LDMC in birch

emphasizes the fast-growing early-successional nature of this species, compared to the other two species (Wright *et al.,* 2004). Stomatal densities showed no treatment effects either. In contrast to the other leaf traits, drought treatment significantly increased leaf chlorophyll content, while metals decreased it. This made leaf chlorophyll content the only leaf trait, besides lead mass and area, that responded to drought and metal stresses in a contrasting manner, as also observed by de Silva *et al.* (2012).

Potential limitations that might influence the results compared to natural situation include the pot size and substrate homogeny. To limit drought stress trees grow deeper roots to find water (Lambers *et al*., 2008). Due to the shallow pot sizes in this study, my trees were unable to do this, and thus were forced to experience intense drought periods. Deep roots also allow trees to maintain a cooler root temperature, as soils are cool compared to warmer surface temperatures. However, pots were placed on stands. In order to limit increasing root temperatures reflective foil was wrapped around the pots to minimize any potential heat stress. Also, natural soils have horizons where most of the contamination is found on the upper layers (Prasad, 2004). This allows for plants to be able to grow roots in the uncontaminated zone and avoid metal contamination. Overall, under natural situations results maybe less extreme.

Conclusions:

Cu and Ni contamination showed a distinct effect on plant-water relationships and plant response to drought in the three investigated broad-leaf tree species red maple (*Acer rubrum*), white birch (*Betula papyrifera*), and red oak (*Quercus rubra*) over a two-year period in lysimeters. Metals reduced stand water consumption and higher substrate moisture in the lysimeters, i.e., metals can have an effect on ecosystem level processes. This has been previously explained as a result of reduced root growth (Menon *et al.,*

2005), which also was observed in the current experiment, but the present results show a more detailed picture. Plant water use was influenced by reduced transpiration rate per leaf area as indicated by the porometer measurements, and by a reduced leaf area per plant. Whether the unused water would result in a higher soil moisture in a natural situation is questionable, however, as losses through evaporation on the soils are likely to increase on a more moist soil. Many of the effects of drought and metal stresses were similar, which has previously been interpreted as metals increasing plant drought tolerance (Disante *et al.,* 2014). However, the lower leaf relative water content (RWC) is a clear indication that the overall effect of metals on plant water relations is negative despite the higher soil moisture content. Future studies are needed to determine whether the drought-stress aggravating effects for metal-contaminated soils could be counteracted in restoration of metal-affected landscapes, for example, by using drought-tolerant species or by improving water-retention capacity of the soils e.g. by addition of organic matter. It would also be worth investigating whether measures that reduce metal phytoavailability, such as liming, would have a positive effect on plant-water relationships.

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Acknowledgements:

I would like to thank Paul and Carol Holla at Holla's Produce and Greenhouse in Blezzard Valley, Ontario Canada for the use for their land and the construction of the greenhouse enclosure over the two-year period. I would like to thank Andrew Tanentzap for all of his statistical advice for this project. I would also like to thank Dan Kirkey for the construction of the large measurement balance, which led to the case of handling and

accuracy of measurements of the lysimeter pots. I would also like to thank Logan Kirkey for all the construction, deconstruction and heavy lifting over the two-year period, and to Shirley Ann Kirkey for helping me collect samples at all hours of the night. Also thank you to Glen Watson, Stephen Monet and the 2012 City of Greater Sudbury Re-greening crew for an efficient clean up. The collaborative research and development grant of the natural science and engineering research council of Canada (NSERC) with Vale and Centre funded this research for Excellence in Mining Innovation (CRDPJ 372568-08). Vale also contributed extra funding towards metal analysis above the grant requirements.

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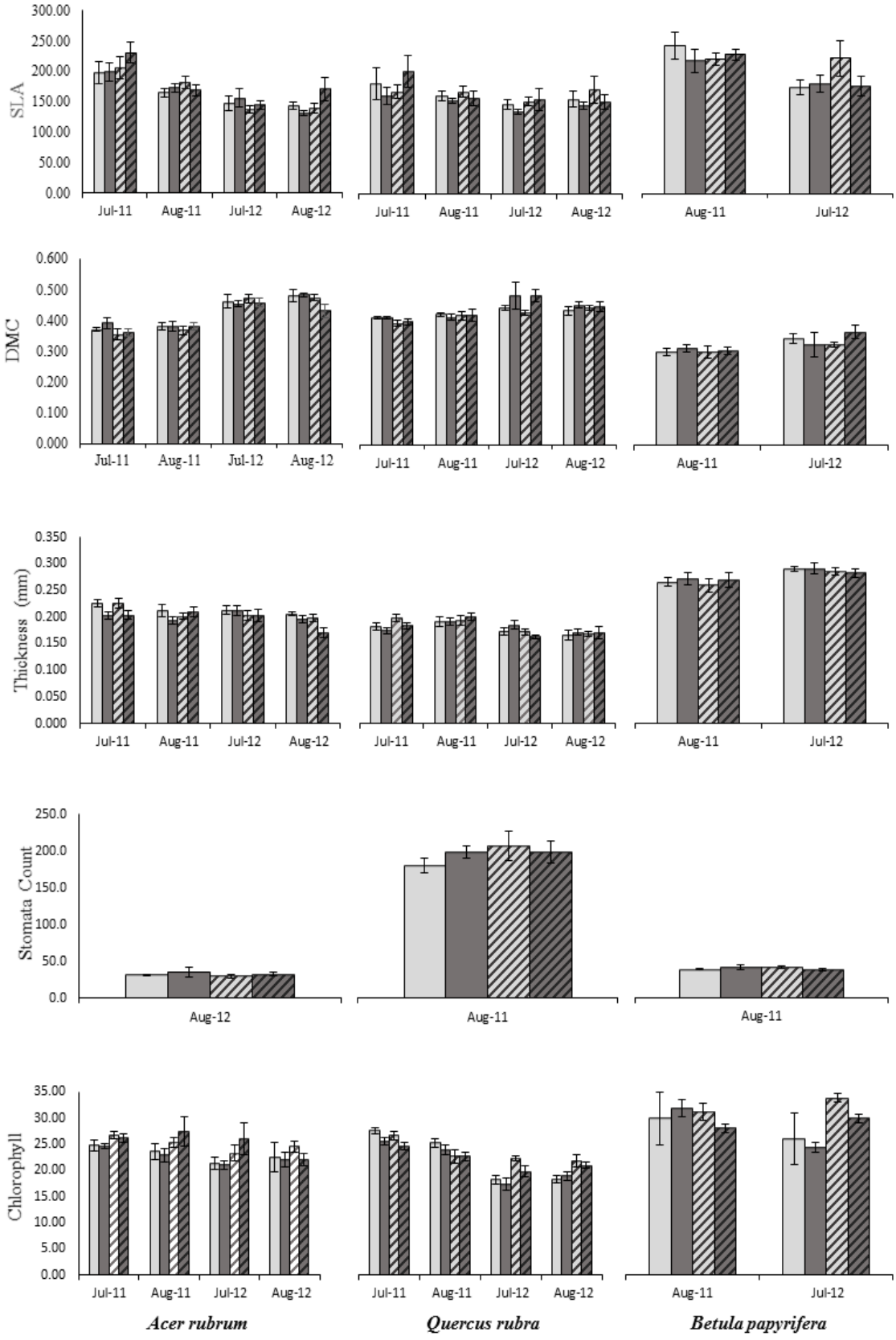
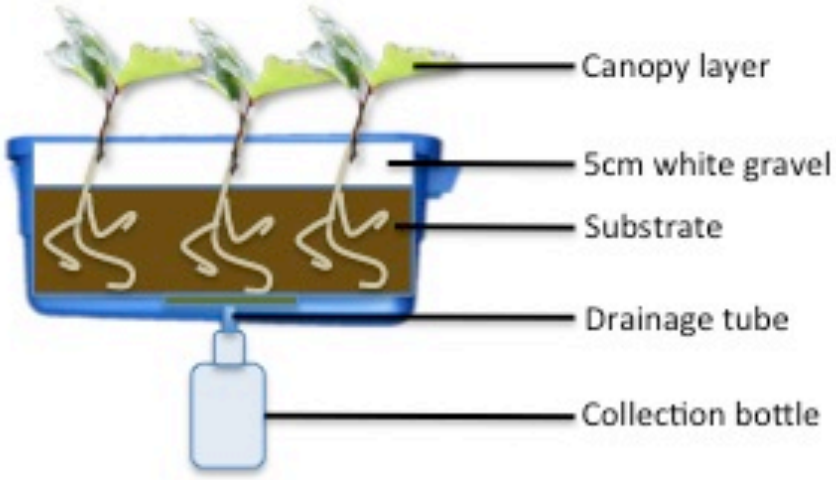

SLA
300.00
250.00
200.00
150.00
100.00
50.00
0.00
DMC
0.600
0.500
0.400
0.300
0.200
0.100
0.000
Thickness (mm)
0.350
0.300
0.250
0.200
0.150
0.100
0.050
0.000
Stomata Count
250.0
200.0
150.0
100.0
50.0
0.0
Chlorophyll
35.00
30.00
25.00
20.00
15.00
10.00
5.00
0.00
Jul-11
Aug-11
Jul-12
Aug-12
Acer rubrum
Quercus rubra
Betula papyrifera

Appendix 2.1a. Mean values of foliar traits (Error bars: ± 1 S.E.) among three species. Traits were: SLA, DMC, leaf thickness (mm), stomata count and chlorophyll content measured in *Acer rubrum, Quercus rubra, Betula papyrifera* in both July and August of 2011 and 2012. Trees were exposed to four different treatments: control: (light fill); metal only: (dark fill); drought only: (light hatched fill); and metal and drought: (dark hatched fill).

Appendix 2.1b. Log-likelihood ratio tests comparing foliar traits among three tree species. Traits were: SLA, DMC, leaf thickness (mm), chlorophyll content, and stomatal count measured in *Acer rubrum, Quercus rubra, Betula papyrifera* in both July and August of 2011 and 2012. Trees were exposed to four different treatments: control (no metal, no drought), drought only, metal only, and metal and drought. Any non-significant interactions or factor are not presented in the table. χ^2 = chi-square statistic; df = degrees of freedom.

Traits	**Treatment**	**χ^2**	**df**	**p-value**
SLA				
	drought	3.30	1	0.069
	species	9.64	2	**0.008**
	harvest	64.33	3	**<0.001**
DMC				
	species	223.74	2	**<0.001**
	harvest	75.71	3	**<0.001**
Thickness (mm)				
	species	245.46	2	**<0.001**
	harvest	19.44	3	**<0.001**
Chlorophyll Content				
	drought	17.89	1	**<0.001**
	species	109.46	2	**<0.001**
	harvest	87.32	3	**<0.001**
	drought × harvest	19.4	3	**<0.001**
		t_{127}		**p-value**
Stomatal Count				
	intercept	69.5		**<0.001**
	metal	1.17		0.245
	drought	0.65		0.520
	species *Betula*	4.51		**<0.001**
	species *Quercus*	37.53		**<0.001**
	metal × drought	-0.98		0.328



Appendix 2.2: Schematic diagram of pot profile, where there is a 5cm white gravel to limit evaporation, the substrate and a one-litre leachate collection bottle underneath the drainage tube.

Appendix 2.3. Particle size distribution of the control substrate (silt loam), and of the pure slag added to the silt loam in the metal treatment. Summary data and percent passing for a given size fraction (µm) are presented. Two replicate samples measured each. MV: Mean Volume Diameter – Mean diameter of the volume distribution. MN: Mean Number Diameter – Mean Diameter of the number distribution. MA: Mean Area Diameter – Mean Diameter of the area distribution. CS: Calculated Specific Surface Area – surface per volume. SD: Standard Deviation - the width of the measured particle size distribution (Geolabs, Sudbury).

Summary data

	MV(µm)	MN(µm)	MA(µm)	CS (mm^{-1})	SD
Loam 1	100.3	7.1	35.4	169	73
Loam 2	77.6	7.2	33.7	178	46
Slag 1	392.6	27.2	171.8	35	290
Slag 2	525.8	60.4	259.0	23	384

Percent passing for a given size fraction (µm)

µm	2830	2380	2000	1680	1410	1190	1000	840	710	590
Loam 1	100	100	100	100	100	100	100	100	99.63	98.9
Loam 2	100	100	100	100	100	100	100	100	100	99.61
Slag 1	100	100	100	100	99.55	98.49	96.17	92.26	84.8	74.92
Slag 2	100	100	99.61	98.73	96.73	93.06	86.88	78.41	69.46	63.06

µm	500	420	350	300	250	210	177	149	125	105
Loam 1	97.76	95.83	93.17	90.85	88.42	86.4	84.45	82.28	79.61	76.29
Loam 2	99.08	98.27	97.11	95.9	94.28	92.55	90.64	88.35	85.51	82
Slag 1	67.72	61.7	55.66	49.81	41.67	33.51	26.53	21.11	17	13.84
Slag 2	59.1	54.54	47.57	39.66	29.96	22.44	17.02	12.98	9.79	7.32

µm	88	74	62.5	53	44	37	31.11	26.2	22.1	18.6
Loam 1	71.99	66.44	59.37	50.95	40.71	31.5	23.82	18.01	13.8	10.67
Loam 2	77.5	71.7	64.28	55.29	44.16	34.05	25.57	19.14	14.48	11.04
Slag 1	11.18	8.91	7	5.46	4.07	3.07	2.28	1.65	1.13	0.7
Slag 2	5.35	3.85	2.74	1.91	1.2	0.7	0.31	0	0	0

µm	15.6	13.08	11.05	9.26	7.8	6.55	5.51	4.63	3.9	3.27
Loam 1	8.27	6.39	4.93	3.69	2.72	1.94	1.31	0.79	0.36	0
Loam 2	8.44	6.45	4.94	3.68	2.7	1.92	1.29	0.78	0.36	0
Slag 1	0.33	0	0	0	0	0	0	0	0	0
Slag 2	0	0	0	0	0	0	0	0	0	0

Appendix 2.4. Pictures illustrating the experimental design (top), and large balance (bottom) underneath the greenhouse plastic covering.

CHAPTER 3

SHORT-TERM AND LONG-TERM EFFECTS OF HEAVY METAL CONTAMINATION ON TRANSPIRATION RATES IN *BETULA PAPYRIFERA*

3.1 Abstract:

The purpose of this study was to investigate to what extent observed negative effects of heavy metals on transpiration rates of young trees are a result of inhibited root water uptake due to acute exposure to metals (short-term effect) or a result of structural damages in plants grown on metal-contaminated substrate (long-term effect). The approach was to investigate differences in responses of transpiration rates of root exposure to heavy metals between plants with and without a previous history of metal contamination. One hundred *Betula papyrifera* seedlings were grown at 2 levels of Co-Cu-Ni contamination for 6 weeks (control vs. 0.2 ppm Co, 6.1 ppm Cu and 5.4 ppm Ni) and transferred into 5 different solutions (control, 28 ppm Ni, 56 ppm Ni , 111 ppm Ni, 28 ppm of Hg) to observe the rate of transpiration. Long-term exposures to the metal-contaminated substrates led to reduced area-based transpiration rates due to smaller leaf area. Acute exposure to Hg reduced transpiration rates per leaf area. Results also indicate that previous exposure to metal contamination may aggravate the negative short-term response to actual Ni exposure, but the results were not significant, possibly due to confounding effects by the large size variation among plants with different growth histories, and the negative relationship between transpiration rate and plant size.

Key Words: Nickel, *Betula papyrifera*, roots, transpiration, heavy metals

***Tanentzap, F.M**., Ryser, P. *Submitted*. Short-term and long-term effects of heavy metals on transpiration of Betula papyrifera..

3.2 Introduction:

Soil contamination with heavy metals has an effect on plant water relationships, as metals influence uptake, transport and loss of water (Poschenrieder and Barcelo, 2004). Uptake of water can be impeded by a low hydraulic permeability of metal-exposed roots resulting from inhibited aquaporin function (Barrowglough *et al*., 2000; Wan and Zwiazek, 1999; Przedpelska-Wasowicz and Wierzbicka, 2011), or by decreased root growth as a result of a reduced root elongation and an altered biomass allocation pattern (Kahle, 1993; Ryser and Emerson, 2007). Transport of water within the plant can be disrupted by blocked xylem vessels and tracheids, or by the smaller vessel size resulting from exposure to metals (Lamoreaux and Chaney, 1977; de Silva *et al*., 2012). Transpiration can be reduced by metals through direct effects on stomata (Yang *et al.,* 2004; Devi *et al.,* 2012), or by increased ABA production in roots, which then causes stomatal closure (Hollenbach *et al*., 1997). The different effects of metals are interconnected. For example, reduction in root hydraulic conductivity is an important factor leading to lower transpiration rates (Wan and Zwiazek, 1999; Rodriquez-Gamir *et al.*, 2010). In field situations, consequences of metal effects on plant water relationships are often aggravated by the drought-prone nature of metal-contaminated landscapes due to soils which can be thin and have low organic matter content (Poschenrieder and Barcelo, 2004).

Most studies on the effects of heavy metals on plant water relations have been limited to short-term experiments, metal effects being assessed within a few days of exposure. Many effects can be observed in a short-term, such as those of Hg, which decreases root permeability within minutes and plant transpiration within hours, followed by a partial recovery after the removal of the contaminant (Maggio and Joly, 1995; Wan

and Zwiazek, 1999; Beaudette *et al.,* 2007). In excised leaves Cd reduced transpiration within minutes (Bazzaz *et al*., 1974). Ni causes K^+ leakage within hours and reduces water content within days, with corresponding recovery times after removal of the contaminant (Llamas *et al.,* 2011). Such short-term reversible effects may be associated with gating of aquaporins (Przedpelska-Wasowicz and Wierzbicka, 2011) and other alterations in membrane characteristics (Llamas *et al*., 2011). Heavy metals such as Cd can also suppress the expression of aquaporin genes (Yamaguchi *et al*., 2009), but an increased expression has been recorded as well, possibly as a response to reduced conductivity due to blocked aquaporins (Beaudette *et al*., 2007). Aquaporins are responsible for the bulk flow of water into the plants, and the slightest change in the intercellular environment can cause aquaporin structure to change and root permeability to be altered (Tyerman *et al.*, 2002), as in case of nutrient shortage (Clarkson *et al*., 2000), anoxia (Tournaire-Roux *et al*., 2003), or heavy metals (Przedpelska-Wasowicz and Wierzbicka, 2011). The fast recovery of aquaporins after removal of the stressor is associated with their fast turnover rate (Maurel *et al*., 2008).

In the present study, within a framework of investigations on heavy metal effects on plant-water relationships, the effect of long-term metal exposure on the extent of short-term responses in whole-plant transpiration was investigated on young trees which had grown for 6 weeks on a contaminated substrate at the beginning of their second growing season, compared to plants that had not been previously exposed to heavy metals. My prediction was that a short-term exposure to heavy metals has a stronger effect on plants that have previously been exposed to such metals, as long-term exposure is known to cause changes in hydraulic architecture (Lamoreuaux and Chaney, 1977; Santala and Ryser, 2009; de Silva *et al*., 2012) or in leaf structure (Molas, 1997), which

can have detrimental effects on the plant's ability to deal with further stress. For example, trees grown on metal-contaminated soil are more likely to suffer embolisms than trees grown on uncontaminated soil (Tanentzap and Ryser, 2015). On the other hand, I also predict that exposure of plant roots to non-contaminated substrate leads to a partial recovery of transpiration due to a recovery of root hydraulic conductivity in the absence of heavy metals in the medium. The aim of this study is to better understand mechanisms of observed reduction in transpiration in trees exposed to heavy metal-containing slag with Ni as the main contaminant (Chapter 2, of this thesis).

The investigation was conducted on young plants of white birch (*Betula papyrifera* L.) grown either on a metal-contaminated or on a uncontaminated substrate. The contamination used for the growth substrate was similar to that caused by the Cu/Ni smelters in the region of Sudbury, ON, where white birch is the dominant tree on sites that have not been reclaimed. I tested the response of transpiration of plants with a previous growth on contaminated and uncontaminated substrates to an acute exposure of their rooting medium with Ni, which has been identified as the main contaminant in the regional soils (Kirkey *et al.,* 2012) and in the growth substrate used. Ni has been shown to accumulate in birch leaves in the region (Theriault *et al.,* 2014). Published reports on the effect of Ni on transpiration are contradictory, some studies finding an effect (Sheoran *et al.,* 1990; Pandey and Sharma, 2002), some not (Bishnoi *et al.,* 1993; Llamas *et al.,* 2008). There seems to be an agreement that Ni has less effect on transpiration than Cd (Sheoran *et al.,* 1990; Pandey and Sharma, 2002). Nevertheless, soil contamination by metals with Ni as the main contaminant has been shown to lead to a distinct reduction of water use by white birch, red maple and red oak (Santala and Ryser, 2009; Chapter 2 in this thesis). In order to test that our experimental setting had the ability to produce

observable results on transpiration via exposure of roots to different solutions, I also included Hg in the experiment, a known inhibitor of water transport via aquaporins (Maurel *et al.* 2008).

3.3 Materials and Methods:

Plant material and growth conditions:

Betula papyrifera seeds were collected in Killarney, Ontario (45°58'05.4"N 81°29'19.9"W) on July 15, 2013, cleaned and germinated immediately outdoors on organic potting soil, where they grew until fall. Leaves of the seedlings senesced naturally in October, 2013 and were brought into a dark, cool (10°C) room before the first frost.

On November 12, 2013, one hundred seedlings were transplanted into individual 460 ml plastic pots after gently removing the potting soil from their roots. Two substrate treatments were applied: as control substrate pure glaciofluvial sand with pH of 5.4 (Paul Lamb, Restoule, Ontario), and as contaminated substrate the same sand mixed with 3% (v/v) finely crushed 2 mm-sieved slag from a Cu-Ni smelter (Vale Ltd Canada, and Fisher Wavy, Sudbury, ON, Canada) (Table 3.1). The sand was medium to coarse with 79% of the grains between 0.25 and 1 mm, with less than 1% silt (<0.05 mm), and a Calculated Specific Surface Area (CS) of 24 $mm^2\,mm^{-3}$. The slag had 3% of the grains <0.05mm and a CS of 22 $mm^2\,mm^{-3}$ (Geoscience Laboratories, Sudbury, Ontario, Appendix 3.1). The pots were arranged in an alternating pattern within a growth chamber (BioChambers, Model: SPC-56, Winnipeg, Manitoba, Canada) with 16 h light of 1000 $\mu mol\ m^{-2}\ s^{-1}$ at 24 °C and 8 h of dark at 16 °C for a 6 week period. Relative humidity was held at 15%. The plants were watered every second day, either with 25 mL of de-

ionized water, or with a nutrient solution containing 3.3 mg N, 1.45 mg P, 2.75 mg K, 0.27 mg Mg, 0.35 mg S, 1.25 mg Ca, 3.5 µg B, 8 µg Cu, 16.5 µg Fe, 8.5 µg Mg, 0.085 µg Mo and 8 µg Zn (Kirkey *et al*. 2012). The positions of the plants were weekly rearranged. The substrate was covered with 2 cm of gravel (Topfin Premium Aquarium Gravel) to minimize evaporation and algal growth.

Effect of acute metal (Ni and Hg) exposure to roots on transpiration:

To measure the effect of acute metal contamination in soil solution on transpiration rates of the plants grown for 6 weeks on the two different substrates, the plants were gently washed out of their growth substrate under low illumination prior to the beginning of the 16 h light period. After that the plants were placed into 15 mL test tubes with different solutions, one in each test tube, and the opening was sealed with parafilm ® to minimize evaporation. The initial mass of each filled and sealed test tube was weighed. Roots were exposed to the solution for 16 h.

The test tubes contained 9 ml of either of 5 different solutions made by mixing 4 ml of nutrient solution with 5 ml of distilled water, or different metal solutions of $NiSO_4$ or $HgCl_2$. The resulting five solutions contained, besides the nutrients, either no additional heavy metals (control; 7.9 pH), or heavy metals as either 28 mg L^{-1} $NiSO_4$, (0.47 mM; 7.0 pH), 56 mg L^{-1} Ni (0.95 mM; 7.1 pH), 111 mg L^{-1} Ni (1.89 mM; 7.3 pH) or 28 mg L^{-1} of $HgCl_2$ solution (0.14 mM; 7.9 pH). The test tubes were arranged in 10 replicate blocks containing one tube of each of the solutions for plants grown either on metal contaminated substrate or on control substrate. Additionally, to quantify evaporation each replicate block contained one test tube with nutrient solution and water, but without a plant. Water loss from test tubes without plants was only 0.6% of the

average water loss from test tubes with plants, indicating that evaporation was negligible compared to transpiration. The test tubes were kept in racks in the growth chamber during the 16-hour light period and re-weighed at the end of it.

Measured plant traits:

After weighing the test tubes for the second time, all leaves were removed and their total leaf area was measured using a Li-3100 Area Meter (LI-COR Inc., Lincoln, Nebraska, USA). Dry mass of all plant parts was measured after drying at 75°C for 48 h. Transpiration rate was calculated as water loss during the 16 h divided by total leaf area.

Soil and Leaf Metal Analysis:

The contents of phytoavailable elements in the growth substrates (weak acid extraction) and the total element contents in leaf tissue were analyzed from samples collected at the end of the experiment, using plasma atomic emission spectroscopy (Testmark Inc., Sudbury, Ontario, Canada). The contaminated growth substrate had significantly higher concentrations of Ni, Co and Cu, compared to the control substrate (Table 3.1).

Statistical analyses:

Plant total leaf area and plant dry mass were analysed with one-way analysis of variance (ANOVA) with growth substrate contamination as independent factor. Plant total transpiration and transpiration rate per leaf area were analysed with two-way General Linear Models with the growth substrate contamination and the type of solution in the test-tubes as factorial variables. When analysing transpiration rates, the total plant

dry mass was used as a covariate, to correct for size effects. All variables were log transformed to attain normality. All analyses were carried out using SYSTAT 12.

3.4 Results:

After six weeks of growth on contaminated substrate*, B. papyrifera* had accumulated a significantly higher amount of Ni and Co, but not Cu, into its foliage (Table 3.1). Plants grown on metal-contaminated substrate had 50% less total leaf area ($F=30.7$, $P<0.001$; ANOVA) and 47% less total dry mass ($F=28.6$, $P<0.001$;ANOVA) compared to plants grown on control substrate. This reduction was associated with a significant 36%-decrease in total transpiration per plant as an average across all metal solutions (Fig. 3.1a, Table 3.2). Among the five treatments of metal concentrations in the test-tubes, plants with roots in Hg solution transpired 44% less than plants in control solution. The Ni solutions reduced total transpiration by 2-6% for plants grown on uncontaminated substrate, and by 10-19% for plants on contaminated substrate. The effect of metal solutions was close to significant ($P=0.075$, Table 3.2). There was no significant interaction between growth substrate and test-tube solution ($F=0.4$, $P=0.833$), and the interaction factor was left out of the final model.

With respect to transpiration rates per leaf area, two plants were obvious outliers, both with a studentized residual >4.3 (Grubbs test $P<0.01$). These plants were not included in the analyses, the outlying values probably being a result of inaccuracy of the determination of leaf area, which for both of these outliers was very small.

There was a negative association between transpiration rate per leaf area and plant size. Hence, total plant dry mass was included in the statistical model as a covariate. The lowest transpiration rates per leaf area were found in test tubes containing Hg, that was

35% less than the rate for the test tubes with control solution (Fig. 3.1b). The effect of the test tube solution was significant (Table 3.2), but the only significant pairwise difference was between the Hg and the control treatments ($P=0.012$; Bonferroni), the other comparisons being non-significant ($P>0.30$). Transpiration rates in Ni-containing tubes were lower for plants grown on contaminated substrate, compared to control plants (Fig.3.1b), but overall the growth substrate did not have a consistent effect, and neither growth substrate nor its interaction with test-tube contents had a significant effect (Table 3.2).

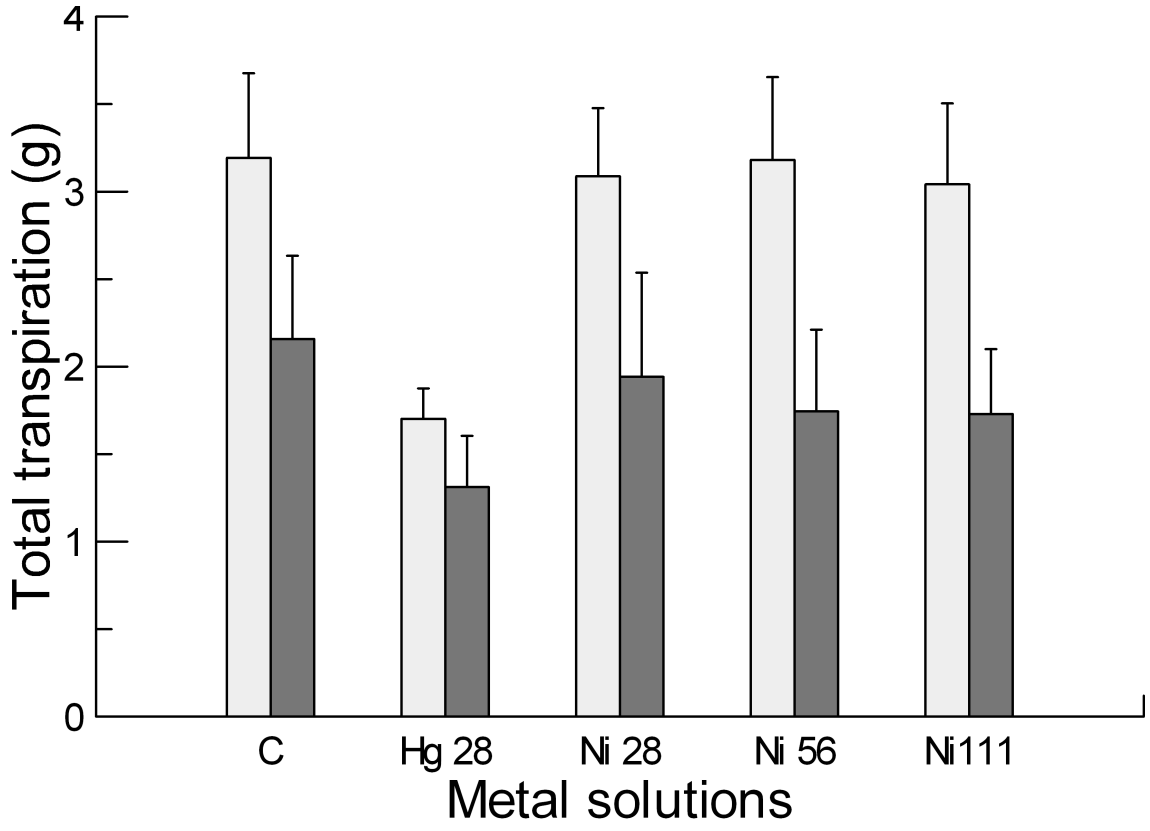


Fig. 3.1. A. Transpiration per plant (g H_20) of *Betula papyrifera* during a 16 h day with roots exposed to 5 different solutions (control (C); 28 ppm Hg; 28 ppm Ni; 56 ppm Ni; 111 ppm Ni) for plants with two growth histories (control= light fill; contaminated with 3% slag= dark fill). Mean values ± 1 SE.

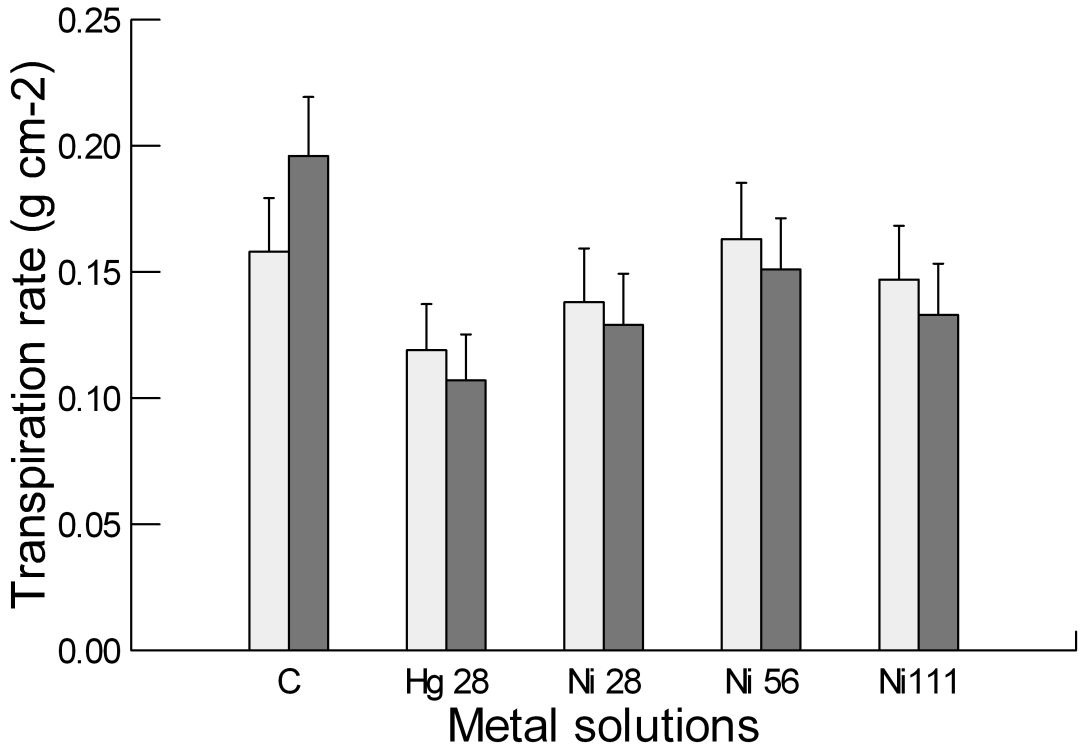


Fig. 3.1 B. Transpiration rate (transpiration leaf area, $g_{H20}\,cm^{-2}$) of *Betula papyrifera* during a 16 h day with roots exposed to 5 different solutions (control (C); 28 ppm Hg; 28 ppm Ni; 56 ppm Ni; 111 ppm Ni) for plants with two growth histories (control=light fill; contaminated with 3% slag=dark fill). Mean values ± 1 SE.

Table 3.1. Phytoavailable using 1 M ammonium acetate for extraction of Co, Cu and Ni in the growth substrates and total Co, Cu and Ni contents in leaves of *Betula papyrifera* (all values in ppm) in the two treatments during growth (0% or 3% slag mixed with sand) (Mean values ± 1S.E, n=3).

Substrate	**Co**		**Cu**		**Ni**	
0%-slag	0.007	±0.003	0.068	±0.001	0.043	±0.002
3%-slag	0.178	±0.077	6.060	±1.018	5.380	±1.848
Krustal-Wallis	**p=0.05**		**p=0.046**		**p=0.05**	
Leaf						
0%-slag	1.897	±0.239	13.133	±0.956	11.353	±0.910
3%-slag	8.893	±1.411	14.000	±1.701	162.067	±37.568
Krustal-Wallis	**p=0.05**		p=0.857		**p=0.05**	

Table 3.2. Results of General Linear Models of total plant transpiration and transpiration rate per leaf area with growth substrate (contaminated and control) and test tube content (28 ppm Hg, 28 ppm Ni, 56 ppm Ni, 111 ppm Ni and Control) as factorial independent variables. Transpiration rate was tested with total plant dry mass as covariate.

Independent variable				**Substrate**	**Tube content**		**Total DM**	
	N	R^2	F	P	F	P	F	P
Total Transp.	99	0.244	21.3	**<0.001**	2.2	0.075	-	-
Transp. rate	96	0.184	0.1	0.808	3.2	**0.016**	6.4	**0.013**

3.5 Discussion:

My data support the opinion that different effects of heavy metals on plant water balance may act on different time-scales (Llamas *et al*., 2011). Both the long-term effects of the plant's growth history on heavy metal-contaminated substrate, and the short-term effects caused by the actual exposure of their roots to metals had an effect on *B. papyrifera* transpiration. Plants grown on contaminated substrate transpired less than control plants in all five investigated actual root environments, but their smaller leaf area was the main contributor to the reduced water use. Besides the reduced leaf area, potential causes for a reduced transpiration could be blocked or reduced xylem vessels diminishing water flow to the leaves (Lamoreaux and Chaney, 1977; de Silva *et al.,* 2012), or some aspects of membrane damage, which may take a longer time to recover. Leakage of K+ from roots of rice in response to Ni toxicity, for example may take days to recover after withdrawal of the contaminant (Llamas *et al.,* 2008). Also, metals may damage guard cells (Molas, 1998), and metals accumulated in the leaves may directly influence stomatal function (Devi *et al*., 2012). In the present study, *B. papyrifera* grown on the contaminated substrate had accumulated Co and Ni in their leaves, as is characteristic for the species in Sudbury region (Theriault *et al*., 2014). But although in the present experiment transpiration rates in plants grown on contaminated substrate were on average lower compared to control plants when exposed to metal solutions in the test-tubes, the differences were not statistically significant. One reason for the inconclusiveness of the results may be the size difference between the plants grown on the different substrates. As transpiration rates per leaf area were lower for larger plants, possibly due to increased boundary layer resistance of the larger leaves (Lambers *et al*.,

2008), differences in transpiration rates among plants grown on different substrates may have been confounded by the variation in size.

The effect of a short-term exposure to Ni^{2+} was less than the effect of exposure to Hg, and not significant. The reduced transpiration of plants with roots immersed in Hg solution, a known aquaporin inhibitor, indicates that in this experiment a short-term effect could be achieved within the 16 hours of exposure of the plants. Consequently, the small effect of Ni indicates that it plays only a minor role that root aquaporins in the plants' responses to this metal. Ni^{2+} slightly reduced transpiration rates in plants grown on contaminated substrate, but error variation was large and the metal × growth substrate interaction was not significant. The low effect of Ni^{2+} on root hydraulic permeability is in agreement with published data, the effect of Ni^{2+}on transpiration being generally less compared to that of Cd (Pandey and Sharma, 2002), or absent (Whiting *et al.,* 2003). But on the other hand, the low effect of Ni^{2+}on transpiration rates observed here is in stark contrast to the massive reduction in transpiration rates in red maple and red oak in a two-year experiment in response to substrate contamination with slag, in which Ni was the main contaminant but present in lower concentrations than in the present study (Chapter 2, this thesis). The concentrations of Ni in the present experiment were similar or higher compared to those observed in contaminated soils in the Sudbury region (SARA Group, 2009), and to concentrations described in published studies where Ni had an effect on transpiration (Pandey and Sharma, 2002). Potential explanations for this discrepancy could be that birch shows a different response to oak and maple, or that the effect can only be observed under drought stress. Also, the effects of Ni^{2+} may need a longer time period to accrue than our current experiment, for example due to increased electrolyte leakage within the roots through modified fatty acid composition and lipid peroxidation

of the membranes (Sanz *et al*., 2009; Gajewska *et al*., 2013). In the 2-year experiment with *Acer rubrum* and *Quercus rubra* with Co, Cu, Ni and Zn treatment, the reduction of stomatal resistance was 6 times more in the second year than in the first year (see Chapter 2 in this thesis). Another possible explanation is other metals present in the two-year experiment, such as Co or Cu, or a combination of all these metals, have a stronger effect on transpiration than Ni alone. Cu was not taken up in the plant leaves, and may have influenced root water uptake by oxidative damage (Ric De Vos *et al*., 1989; Thounaojam *et al*., 2012). Co has been found to reduce transpiration, but for Cu the published results are contradictory (Chatterjee and Chaterjee, 2000; Ahmad *et al*., 2008; Musante and White, 2010). Nevertheless, even without an effect on transpiration Ni has frequently been shown to reduce plant water content in experimental situations (Llamas *et al*., 2008).

I conclude that a short-term response of root exposure to elevated Ni contamination hardly contributes to the previously observed massive reduction in transpiration rates of young *Betula papyrifera* on Ni/Cu/Co contaminated substrate, but a long-term damage may amplify this effect. The reduced water use by plants grown for 6 weeks on heavy metal containing substrate could be explained by their reduced leaf area. There was some indication that damage caused by long-term exposure to metals may influence the short-term response in water uptake, but the results were not conclusive because the observed trends on transpiration rates were not statistically significant, probably due to the large size variation among the plants, which also affects transpiration rates. Understanding the effects of metal contamination at different temporal scales helps to create a greater understanding of how plant-water relationships at the whole plant level are influenced by heavy metal contamination.

Acknowledgements:

I would like to thank Dr. John Gunn for the use of the growth chamber, Dr, Andrew Tanentzap for help with seed collection and measurements and Tarra Degazio for preliminary testing of the method. This research was funded by a Collaborative Research and Development grant of the Natural Science and Engineering Research Council of Canada (NSERC) with Vale and Centre for Excellence in Mining Innovation (CRDPJ 372568-08).

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Appendix 3.1. Particle size distribution of the control substrate (silt loam), and of the pure slag added to the silt loam in the metal treatment. Summary data and percent passing for a given size fraction (µm) are presented. Two replicate samples measured each. MV: Mean Volume Diameter – Mean diameter of the volume distribution. MN: Mean Number Diameter – Mean Diameter of the number distribution. MA: Mean Area Diameter – Mean Diameter of the area distribution. CS: Calculated Specific Surface Area – surface per volume. SD: Standard Deviation - the width of the measured particle size distribution (Geolabs, Sudbury).

Summary data

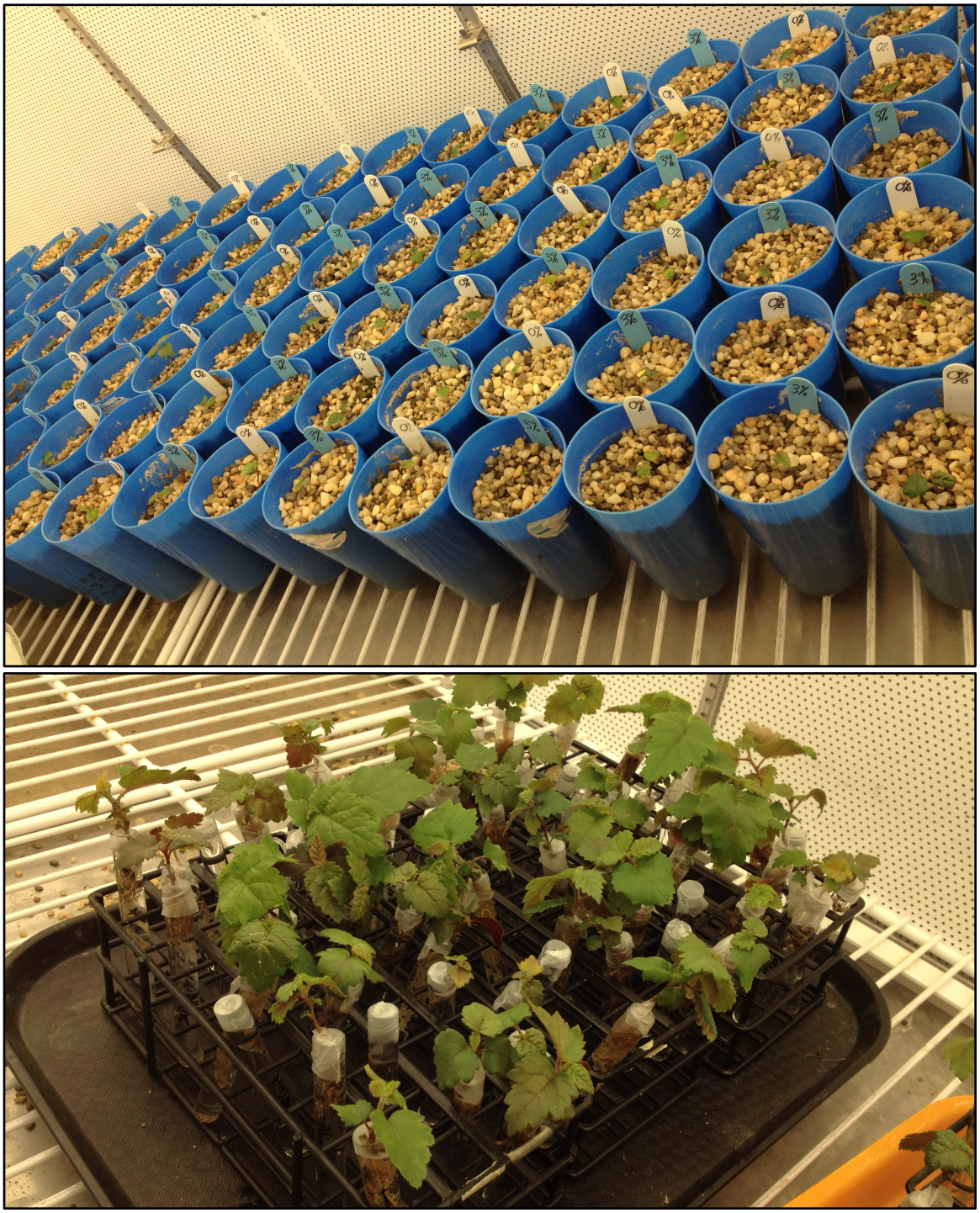
	MV(µm)	MN(µm)	MA(µm)	CS (mm^{-1})	SD
Loam 1	302.4	103.2	219.9	27	158
Loam 2	378.2	164.5	300.3	20	169
Slag 1	492.7	37.8	254.2	24	304
Slag 2	677.0	28.7	287.5	21	469

Percent passing for a given size fraction (µm)

µm	2830	2380	2000	1680	1410	1190	1000	840	710	590
Loam 1	100	100	100	100	100	100	100	99.72	98.695	95.28
Loam 2	100	100	100	100	100	100	100	99.33	97.41	90.15
Slag 1	100	100	100	100	99.61	98.23	94.48	87.66	75.33	61.71
Slag 2	100	99.65	98.91	96.83	92.12	84.85	75.59	67.01	59.6	52.6

µm	500	420	350	300	250	210	177	149	125	105
Loam 1	88.58	77.71	64.87	54.53	43.35	33.42	24.47	16.81	10.845	6.76
Loam 2	77.13	60.51	45.33	35.22	25.31	17.17	10.66	6.04	3.21	1.68
Slag 1	53.21	46.53	39.93	33.81	26.21	19.69	14.83	11.36	8.86	7.02
Slag 2	46.04	37.92	29.13	22.94	17.57	13.95	11.28	9.16	7.44	6.08

µm	88	74	62.5	53	44	37	31.11	26.2	22.1	18.6
Loam 1	4.06	2.35	1.29	0.63	0.16	0	0	0	0	0
Loam 2	0.83	0.33	0.01	0	0	0	0	0	0	0
Slag 1	5.56	4.41	3.48	2.73	2.04	1.51	1.06	0.67	0.33	0.01
Slag 2	4.98	4.11	3.41	2.82	2.25	1.79	1.37	0.99	0.65	0.32



Appendix 3.2. Pictures illustrating the experimental design of long-term (top), and short-term (bottom) of white birch trees.

CHAPTER 4

DECREASED RESISTANCE TO EMBOLISM IN RED MAPLE (*ACER RUBRUM*) SAPLINGS WITHIN A HEAVY METAL CONTAMINATED REGION

4.1 Abstract:

The effects of smelter-damaged environment on embolism resistance in red maple (*Acer rubrum* L.) were investigated with stems of saplings collected *in situ* from within either the metal-contaminated (mainly Cu and Ni) region of Sudbury, Ontario, or from within the uncontaminated surrounding regions. Current levels of aerial pollution in all regions are low. Stem hydraulic conductivity was measured after stems were flushed for 40 minutes and then being spun using the centrifuge technique at -3 MPa. Red maple saplings from within the metal contaminated regions had about twice the percent loss of conductivity, due to embolisms, as saplings from outside the contaminated area. Sapling vulnerability to embolism shows contrasting trends with age in contaminated and non-contaminated regions. The increased vulnerability of trees to catastrophic xylem dysfunction under drought may contribute to the observed stunted growth and periodic stem-dieback of trees in metal-contaminated landscapes, the effects being exacerbated by low water-holding capacity of the thin soils as a consequence of the erosion when the vegetation was damaged.

Key words: embolism; heavy metals; *Acer rubrum*; Cu; Ni; stem dieback

* **Tanentzap, F.M**., Ryser, P. 2015. Decreased resistance to embolism in red maple (*Acer rubrum* L.) saplings within a heavy metal contaminated region, *Environmental and Experimental Botany*. 109:40-44.

4.2 Introduction:

Heavy metal contamination is known to reduce plant growth and to influence their growth form (Prasad, 2004). Vegetation on ultramafic soils with naturally high metal concentrations such as Cr, Co and Ni often shows stunted growth compared to neighbouring sites, with substrate toxicity and lack of nutrients being suggested as possible reasons (Jacobson, 1968; Chiarucci *et al*., 1998; Enright *et al*., 2001). A stunted tree canopy can also be observed in contaminated environments around non-ferrous smelters (Kozlov *et al*., 2009), well-studied examples being *e.g.,* around the Cu-Ni smelters in Sudbury, Canada (James and Courtin, 1985; Winterhalder, 1996) and on the Kola Peninsula, Russia (Kryuchkov, 1993). Trees in metal-contaminated landscapes commonly show periodic stem dieback, leading to growth forms with multiple stems (James and Courtin, 1985; Kruychkov, 1993; Dickinson *et al*., 1996; Zverev *et al*., 2013).

Stem and branch dieback in trees and tree seedling mortality are often a result of a catastrophic hydraulic failure due to embolised xylem vessels (Tyree and Sperry, 1988; Sperry *et al.,* 1988; Davis *et al*., 2002). Such embolisms are induced by external factors such as drought, freezing, or pathogens (Tyree and Sperry, 1989), but their likelihood is influenced by plant-internal factors such as twig age (Ladjal *et al*., 2005), xylem vessel size (Sperry *et al*., 1994) and pit structure (Hacke and Jansen, 2009). Damage to xylem structure caused by previous drought, for example, can increase vulnerability to cavitation (Hacke *et al.,* 2001b). Metal contamination of the growth substrate is known to affect xylem structure, e.g., by leading to a build-up of blocked xylem vessels in seedlings of *Acer saccharinum* L. and *A. rubrum* L. (Lamoreaux and Chaney, 1977; de Silva *et al.,* 2012). If metal-induced changes in xylem structure increase the risk of cavitation, it is possible that the frequent stem dieback and multi-stemmed growth-form

of trees in the metal-contaminated regions could be explained by susceptibility to hydraulic failure.

In the present study, I measured the susceptibility to embolisms of stems of red maple (*Acer rubrum* L.) saplings collected *in situ* from either the metal-contaminated region of Sudbury, Ontario, or from neighbouring non-contaminated regions, hypothesizing that stems collected in the contaminated area will show a more pronounced loss of conductivity when water stress is applied on the stems, compared to stems collected in non-contaminated regions. All the above-mentioned published studies of the effects of metals on xylem were conducted under experimental conditions with young seedlings. Little to no research has been done on drought tolerance on plants actually grown in metal-contaminated landscapes, and the actual vulnerability to cavitation has not been investigated. Our goal is to gain information to what extent trees growing in a metal-contaminated area are susceptible to other stresses such as drought, compared to uncontaminated regions. Understanding the factors influencing susceptibility of trees in metal-contaminated environments to other environmental stresses could help to optimize the success of restoration efforts of metal-contaminated habitats by allowing an appropriate species choice, and possibly introducing mitigating measures to reduce the stresses.

4.3 Materials and Methods:

Study species and origin of plants:

Red maple (*Acer rubrum* L.) is a diffuse-porous deciduous tree species commonly found across a wide range of habitats in eastern North America (Farrar, 1995). In the Sudbury region, red maple is one of the species which survived for close to a century of

severe air and soil pollution from Cu-Ni smelting, but did so only by expressing an atypical multi-stemmed growth form with frequent stem die-back (Amiro and Courtin, 1981). For the present study stems of red maple saplings were collected in central Ontario in the Great Lakes-St. Lawrence ecotone, from six sites within the metal-contaminated region around Sudbury, and in six sites within uncontaminated neighboring regions (four sites in Powassan 130 km east of Sudbury and two sites in Webbwood 75 km west of Sudbury; Kirkey *et al*., 2012). All stems were collected under a developed canopy. All sites in the contaminated Sudbury region were selected from areas which had not been exposed to restoration efforts by liming, seeding or tree-planting (Lautenbach *et al*., 1995). The sampling sites were the same ones as used in Kirkey *et al.* (2012), in order to have data on soil metal contamination. In that work the sites were selected by the presence of fruit-carrying red maple trees.

The six sites in the Sudbury region have significantly higher concentrations of phyto-available soil metals, compared to the sites in the neighbouring regions, measured using $LiNO_3$ extraction (Abedin and Spiers, 2006) and ICP-AES. The concentrations of Cu and Ni were the highest with 1400 and 3237 ppb in the Sudbury region, compared to 17 and 25 ppb in the neighbouring regions, respectively, but also Co and Cd had significantly elevated levels compared to the neighbouring regions (Kirkey *et al.,* 2012).

The studied regions are similar with respect to climate. Annual average temperature is 4.1 °C in Sudbury, compared to 4.7 °C in Powassan and 4.3 °C in Webbwood (weather station Massey 16 km ESE of Webbwood), the differences being the largest in the winter. Average annual precipitation slightly increases from west to east with 890 mm in Massey, 903 mm in Sudbury and 950 mm in Powassan, with the largest

differences during the months May to September (Environment Canada, http://climate.weather.gc.ca).

Vulnerability to cavitation:

Stem segments of red maple saplings were collected on 6-7 September, 2013. Height of the collected stems ranged from 70 to 120 cm and their diameter at the mid-section from 4 to 8 mm. All stems were from single-stemmed individuals with no stem dieback. Three replicate saplings from each of the 12 sites – 6 metal-contaminated and 6 uncontaminated – resulted in a total of 36 collected tree saplings. Two saplings were discarded due to an error at collection. The collected stems were wrapped with moist paper towels and sealed tightly in large plastic bags for transport. The samples were transported to the laboratory at the University of Alberta in Edmonton. In the laboratory, 3 days after collection, all stems were cut under water to a standard length of 142 mm. Pairs with similar diameters – one sample from non-contaminated and contaminated sites each – were selected to minimize unbalancing in the centrifuge. The segments were selected in positions with minimal branching, and the few remaining leaves were removed. Any branches were cut and the branch collar then wrapped with plastic paraffin film. Both ends of each stem segment were cut cleanly with a razor blade. Prepared samples were flushed for 40 minutes at 1 bar with a 1 mM $CaCl_2$ and 20 mM KCl solution to remove any native embolisms and embolisms developed during the transport (Sperry *et al.,* 1988). Native embolisms were not measured, as such measurements would not have been reliable due to the delay between sample collection and measurements in the laboratory.

Flushed segments were placed into an apparatus as described by Sperry *et al.* (1988) and their hydraulic conductivity (K_{max}) was measured using a Sartorius CP 225 D balance (Sartorius AG, Göttingen, Germany) that was connected to a computer. Hydraulic conductivity (K_{max}) was calculated as:

$$K_{max} = F * (L / \Delta P) \quad (1)$$

where F is the flow rate (g/s), L is the length (mm) of the segment and Δ P is the difference in pressure (MPa).

The centrifuge technique, described by Pockman *et al*. (1995) and Alder *et al*. (1997), was used to measure xylem vulnerability to cavitation. After the initial maximum conductivity was measured, the flushed stem segments were placed into a Sorvall RC-5C PLUS centrifuge (Sorvall, Newton, CT, USA) for 10 minutes at a target pressure of -3 MPa . After spinning, the segments were re-measured for hydraulic conductivity (K). The percentage loss of hydraulic conductivity (PLC) was calculated as:

$$PLC = 100(1-K/K_{max}) \quad (2)$$

where K is the xylem conductivity after the segment had been spun in the centrifuge (Cochard, 2006).

Anatomical measurements:

Free-hand cross sections of the stems were made using a razor blade, and micrographs recorded using a digital camera in a dissection microscope (Leica EZ4D, Heerbrugg, Switzerland) at ×40 magnification along with a scale. Xylem area was measured using Image J 1.46r (Wayne Rasband, National Institute of Health, USA) for PC. The age of each stem segment was determined by counting the annual rings. Stem diameters were measured with a caliper at the midsection of each segment.

Specific hydraulic conductivity (conductivity per xylem area; K_x) was calculated by dividing the maximum hydraulic conductivity after flushing (K_{max}) by total xylem area (Lens *et al.,* 2011).

Statistical analysis:

All measured and calculated variables were checked for normality using Shapiro-Wilcox test. PLC and K_{max} were log-transformed to attain normality. In two stems the measured K after spinning was about 200% larger than K_{max}, indicating artifacts. These two stems were deemed as outliers (studentized residual < -4; Grubb's statistic>3.4, $p<0.05$) and were not included in the analyses.

Differences in measured variables between stems collected at contaminated and non-contaminated sites were tested with nested General Linear Models with site contamination as grouping factor (2 levels) and the sites of collection as subgroups (12 sites), nested within the contamination factor. The effect of stem age on the vulnerability to embolisms was tested by the same model for including the age as a continuous variable, and an interaction factor between contamination and stem segment age. Correlations between all measured variables were tested using Pearson correlation with Bonferroni correction for multiple testing. Correlation between soil metal contamination and PLC was tested using Spearman Rank Correlation tests. Concentrations of the two most important contaminants, Cu and Ni were used as indicators of soil contamination at the sites (Kirkey *et al.,* 2012).

4.4 Results:

After stem segments had been spun at -3 MPa to induce embolism, the percentage loss of conductivity was significantly higher for trees from the contaminated Sudbury region than for trees from neighbouring non-contaminated regions (Table 4.1). While trees from neighbouring areas lost about 20% of their conductivity after being spun, the PLC for those from the Sudbury region was twice as high, at around 40%.

There was no significant difference between the contaminated and non-contaminated regions with respect to any other measured stem characteristic (Table 4.1). K_x was 14% smaller in the trees from the contaminated region than in those from the non-contaminated region, but in contrast to our hypothesis, the difference was statistically not significant.

K_{max} was positively related to stem diameter and xylem area, but there were no significant relationships between morphological stem characteristics and PLC (Table 4.2). Age of the measured stems did not have a general effect on the PLC, but there was an interaction between stem age and contamination factor with respect to PLC, which was close to significance ($p=0.052$; Table 4.3). In stems from the contaminated region PLC had a decreasing trend with age, whereas for stems from non-contaminated regions the trend was a slight increase in PLC with age (Fig. 4.1).

PLC of the stems increased with increasing degree of soil contamination at the site of stem collection. Average PLC of the sites correlated positively (Spearman Rank Correlations, N=12) with the ranks of the sites with respect to contamination by Ni ($R=0.706$; $p=0.036$), and by Cu ($R=0.739$; $p=0.066$).

Table 4.1. Mean values (± SE) of stem age, stem diameter, xylem area, stem maximal conductance (K_{max}), stem specific conductivity (K_x), and percentage loss of conductance (PLC) of *Acer rubrum* saplings and results of General Linear Models of these variables with the contamination at region of origin and site, nested within the contamination factor, as independent variables. K_x and PLC were log transformed to attain normality (N=32).

Measured Traits	**Mean values**		**R^2**	**F**	**p**	**F**	**p**
	Non-Contaminated	Contaminated		Contamination of region		Site	
Age (years)	4.6 ± 0.5	5.1 ± 0.6	0.387	0.74	0.399	1.21	0.342
Stem diameter (mm)	6.3 ± 0.3	6.2 ± 0.4	0.640	2.39	0.414	3.52	**0.008**
Xylem area (mm^2)	22.9 ± 2.2	21.8 ± 2.0	0.563	1.66	0.212	2.55	**0.036**
K_{max} (mg mm s^{-1} MPa^{-1})	22.6 ± 2.7	21.2 ± 4.1	0.229	0.53	0.473	0.59	0.806
K_x (mg s^{-1} MPa^{-1} mm^{-1})	1.04 ± 0.13	0.89 ± 0.13	0.295	0.73	0.402	0.77	0.653
PLC (%)	22.3 ± 4.9	41.3 ± 6.9	0.447	4.82	**0.040**	1.09	0.411

Table 4.2. Correlations between stem age, stem diameter, xylem area, stem maximal conductance (K_{max}), stem specific conductivity (K_x), and percentage loss of conductance (PLC) of *A. rubrum* saplings. Standardized Pearson correlation coefficients (n=32) and Bonferroni-corrected probabilities given if $p<0.05$ (*$p<0.01$; *** $p<0.001$).

	Stem age	Stem diameter	Xylem area	K_{max}	K_x
Stem diameter	0.20				
Xylem area	0.32	0.83***			
K_{max}	0.16	0.64***	0.61***		
K_x	-0.06	0.25	-	-	
PLC	0.01	-0.04	0.01	-0.08	-0.10

Table 4.3. Results of a General model with PLC as dependent variable, the contamination at the region of origin and site, nested within the contamination factor, as independent factorial variables, and stem age as an independent continuous variable. Interaction of contamination factor and age was included in the model.

	R^2=0.577	
	F	p
Contamination at origin	7.70	0.013
Site	1.32	0.294
Age	0.02	0.868
Contamination × Age	4.31	0.052

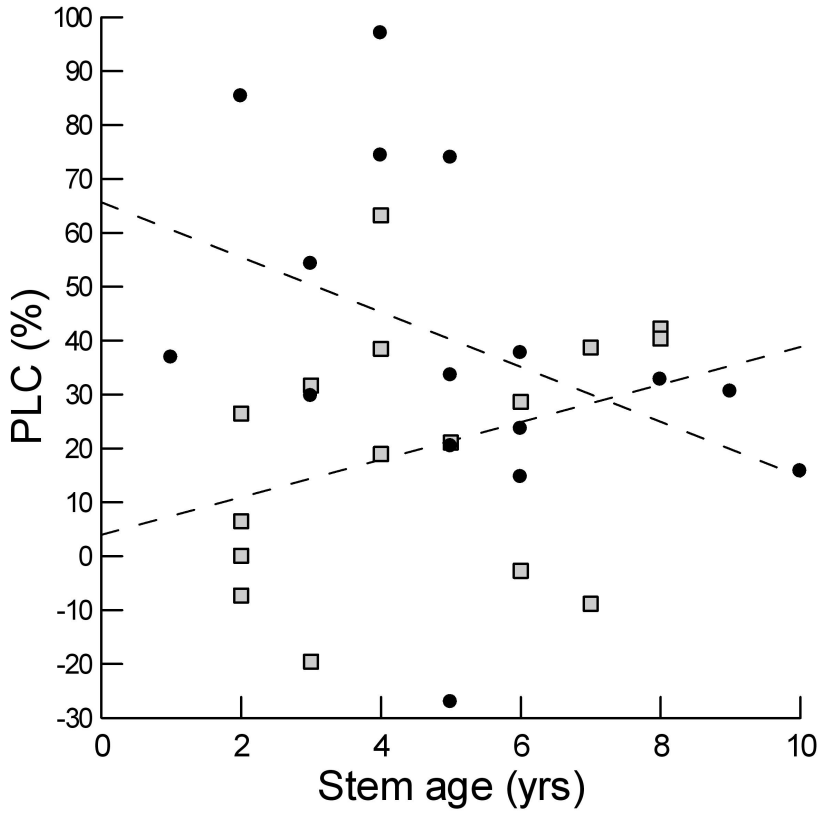


Fig. 4.1. Percentage loss of conductivity (PLC %) of *A. rubrum* stems plotted against stem age, in non-contaminated sites (light-grey squares) and on contaminated sites (dark circles). Dashed lines represent regression lines.

4.5 Discussion:

Red maple saplings growing in the Sudbury region, severely affected by Cu-Ni smelters in the past, were more vulnerable to embolisms than saplings growing in the neighbouring regions. The most pronounced current difference between environmental conditions of these regions is the high soil metal contamination in the Sudbury region (SARA Group, 2008), concentrations of available metals at our study sites around Sudbury being about a 100-fold of those in the neighbouring regions. Air quality in Sudbury has improved since the 1970ies (Potvin and Negusanti, 1995) and is nowadays well within the provincial criteria (AECOM, 2014). All saplings were collected under a developed canopy on sites where red maple is known to produce fruit. The canopy in the Sudbury region are still thinner than in the neighbouring regions due to erosion when the vegetation was damaged, and the canopy somewhat more open. However, there is no evidence in the literature that such conditions would make trees more vulnerable to embolisms. On the other hand, published data shows effects of metals on xylem structure, which may account for the observed results.

Risk of embolism depends on xylem structure (Hacke and Sperry, 2001), and metal contamination has previously been shown to influence xylem structure in this species, resulting in smaller xylem vessels and correspondingly, in a lower K_x, a response which was similar to that caused by water limitation (de Silva *et al*., 2012). Smaller vessels and a lower K_x are actually known to increase a plant's drought tolerance and decrease its vulnerability to cavitation (Sparks and Black, 1999; Mitchell *et al.,* 2008; Cai and Tyree, 2010), but the observed increased risk of embolisms of red maple saplings on metal-

contaminated soils indicates that metal contamination also has other, detrimental effects on xylem structure.

Drought-induced cavitation either results from air seeding via bordered pits (Zimmermann, 1983), or from conduit implosion (Hacke *et al*., 2001a). Vulnerability to air seeding is related to mechanical properties of pit membranes (Hacke and Sperry, 2001), damage to these membranes increasing the risk (Hacke *et al*., 2001b). In order to prevent implosion, xylem conduits need to be reinforced and have lignified cell walls (Hacke and Sperry, 2001), defects in cell walls increasing the risk of implosions (Hacke *et al.,* 2001a).

Exposure to heavy metals has been found to lead to changes in xylem wall structure. In beans, Cd exposure results in accumulation of phenolics and clotted depositions on xylem walls (Fuhrer, 1982; Barceló *et al*., 1988). Zn exposure of this species leads to altered primary walls and middle lamella, especially at the pit membranes, and leads to deposits in the secondary walls (Robb *et al*., 1980). Lamoraux and Chaney (1977) describe a Cd treatment of *Acer saccharinum* leading to xylem vessels blocked with cellular debris and gums, associated with stunted growth and wilting of the plants. In *Pinus sylvestris* roots, Cd has been described to lead to hastened xylem lignification (Schützendübel *et al.*, 2001). In red maple clogged vessels were observed in response to treatment with Cu and Ni containing slag (de Silva *et al*., 2012). These observed changes in xylem cell wall structure in response to metals may reduce the vessel hydraulic safety and affect the susceptibility to cavitation.

Embolisms are known to be associated with branch mortality in *Quercus petraea* (Cochard *et al*., 1996) and in *Populus* species (Rood *et al*., 2000). An increased vulnerability to cavitation could explain the high branch mortality and multi-stemmed

growth forms observed in metal-contaminated environments such as in the Sudbury region for *Quercus rubra*, *A. rubrum* and *Betula papyrifera* (James and Courtin, 1985), and in northern Russia for *B. pubescens* ssp. *czerepanovii* (Kruychkov, 1993; Zverev *et al*., 2013) and for *Salix* species (Zvereva and Kozlov, 2001). The decreasing PLC with increasing stem age for the saplings from the contaminated substrate could be explained by susceptible stems not reaching an old age.

Soil contamination by toxic metals, either by natural (Kayama *et al*., 2002) or anthropogenic causes (Ranta *et al*., 1994), tends to be associated with shorter trees compared to non-contaminated areas. In the case of serpentine soils, the short stature of trees has been suggested to be associated with soil nutrient deficiency (Chiarucci *et al*., 1998), and also mine and smelter-affected sites tend to have low soil nutrient availabilities (Helmisaari *et al*. 1995; Cooke and Johnson, 2002). However, tree height is also limited by plant hydraulic architecture, water potential decreasing with tree height (Bauerle *et al*., 1999). Xylem vessel resistance to cavitation increases towards the top of the tree (Woodruff *et al*., 2008), the limits of this adjustment determining the maximal tree height (Domec *et al*., 2008). Increased vulnerability to cavitation as a result of defective xylem structure might hence contribute to the observed stunted growth of trees in heavy metal contaminated environments.

Airborne emissions such as SO_2 and metal particulates are considered to have led to environmental devastation around smelters such as in the Sudbury area (Hutchinson and Whitby, 1977) and in northern Russia (Zverev, 2009). The current atmospheric pollution levels in Sudbury are unlikely to be responsible for the observed results (AECOM, 2014), but soil metal levels remain high (SARA Group, 2008). The observed high vulnerability to cavitation in saplings from this region, and the ongoing branch die-back in red maple,

red oak and white birch (Fallon Tanentzap and Peter Ryser, personal observations), indicate that elevated soil metal contamination is an important factor contributing to the current damage of the trees. This is supported by the observed correlation between the measured soil metal levels and the percentage loss of conductivity in the saplings. Likewise, in northern Russia birch stem mortality was not reduced despite improvement in air quality (Zverev, 2009). Even relatively low concentrations of toxic metals may result in increased branch mortality, as across southern Ontario *A. saccharum* crown condition is negatively associated with soil metal concentrations, even though all concentrations remain below published critical values (Watmough, 2010).

I conclude that heavy metal contamination in soil increases vulnerability of trees to catastrophic xylem dysfunction under drought, exacerbated by the thin soils coupled with low nutrients and drying out. This could be a factor contributing to the observed stunted growth and periodic stem-dieback with coppiced appearance of trees in metal-contaminated landscapes. As a possible way to mitigate these effects, use of drought-adapted species or genotypes in restoration of metal-impacted areas should be investigated.

Acknowledgements:

I would like to thank Dr. Uwe Hacke and members of his lab at the University of Alberta for the help with understanding the methodology and for the use of his laboratory equipment to measure hydraulic conductivity and embolism resistance. This research was funded by the Collaborative Research and Development grant from the Natural Sciences and Engineering Research Council of Canada (NSERC) with Vale Canada Ltd. and the Centre for Excellence in Mining Innovation (CRDPJ 372568-08).

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Appendix 4.1. Pictures illustrating the tree collection sites: contaminated site (top), non-contaminated site (bottom).

CHAPTER 5

MEASUREMENT OF *IN SITU* LEAF RELATIVE WATER CONTENT (RWC) OF FIELD-GROWN PLANTS

5.1 Abstract:

Premise of the study: Investigation of constraints associated with measuring leaf Relative Water Content (RWC) in field-grown plants, posed by water loss and other changes in leaves during transport from field to laboratory.

Methods and Results: For four contrasting species (*Alnus incana*, *Comptonia peregrina*, *Impatiens capensis*, *Scirpus microcarpus*) the influence of duration of storage up to 96 hours and storage temperature on leaf RWC and its components was investigated. After 24 hours in cool (10°C) storage RWC remained within 5% of the initial value. Both leaf fresh mass and saturated mass decreased over time. The effect of storage depended on species identity and was stronger under warm conditions.

Conclusions: The study confirms that this method enables measurement of RWC for field-grown plants with reasonable accuracy, but care has to be taken with species vulnerable to desiccation. Efficient use of cooled space and low tare mass of plastic bags are advantages compared to other types of containers. Other leaf traits, such as leaf dry matter content, appeared to be more sensitive to storage than RWC.

Key Words: Field measurement; Plant Water Status; Relative Water Content

* **Tanentzap, F.M**., Stempel, A., Ryser, P. *Submitted*. Measurement of in situ leaf relative water content (RWC) of field-grown plants.

5.2 Introduction:

The Relative Water Content (RWC) of leaves is frequently used to describe plant water status at a given time (Barrs, 1968; Kramer, 1969). It is a sensitive variable, that responds to environmental conditions such as temperature, light, humidity and water supply (Slatyer, 1962). It is defined as the percentage of water present at the time of sampling relative to the amount of water in a saturated leaf, calculated as

$$RWC\ (\%) = 100 \times (FM - DM)/(SM - DM) \qquad \text{(Eq. 1)}$$

where, FM is leaf fresh mass at the time of collection, SM leaf mass at saturated condition, and DM leaf dry mass (Turner, 1981). Accuracy of the measurement of the saturated mass has received considerable attention; the most commonly used method to achieve saturation being floating punched leaf circles on water (Stocker, 1929; Weatherley, 1950; Barrs and Weatherly, 1962; Slatyer, 1967; Barrs, 1968; Kramer, 1969; Smart and Bingham, 1974; Gonzalez and Gonzalez-Vilar, 2001). Protocols to saturate leaves, useful for large scale comparative measurements of field-grown plants have recently been described by Garnier *et al.* (2001) and Ryser *et al.* (2008), the former by rehydrating leaves by placing the twigs in water-filled tubes after 12 h, the latter by storing the leaves between moist paper towels for 12h.

Constraints influencing the measurement of the initial fresh mass have received less attention, as in most of the cases leaf relative water content has been assessed in the laboratory, where the fresh mass of the collected leaves could be directly measured. In the field, accurate balances are not easily available, and as the excised leaves quickly lose water, collecting leaves in the field requires protective measures against water loss. Stocker (1929) and Weatherley (1950) collected leaves in metal or glass containers, or wrapped them in impermeable tissue. Hadley and Smith (1983) used plastic vials for

conifer needles, and found that there was no significant difference for measurements after 4 h and 48 h. Smart and Bingham (1974), Gonzalez and Gonzalez-Vilar (2001), Ryser *et al.* (2011), Walter *et al.* (2011) and Teszlak *et al.* (2013) used sealed plastic bags for transportation from field to laboratory. Armas *et al.* (2010) and Aref *et al.* (2013) used microcentrifuge tubes or other polyethylene tubes for coniferous leaves. Govender *et al.* (2009) stress the importance of a proper sampling procedure to prevent any water loss by keeping leaves in plastic bags and in a cool dark place, but the effects of the duration of transportation and conditions during the transport on water loss have never been quantified. The method used is often not even described. Breashers *et al.* (1997) conclude that a one-hour storage of coniferous leaves in plastic bags kept in cool and dark does not change leaf characteristics. Besides water loss, accuracy of the calculation of RWC can also be affected by cellular respiration (Gonzalez and Gonzalez-Vilar, 2001), which may decrease leaf dry mass during storage at warm temperatures, and storage conditions may influence membrane characteristics determining water content at saturation (Deschene *et al.,* 1991). Thus, the duration and conditions of transportation of leaves from field to laboratory have to be given careful consideration when assessing RWC of field-grown plants.

The purpose of this investigation was to determine constraints for accurate measurements of RWC, caused by transportation from the field to a laboratory. I investigate the effects of both duration and temperature of storage on leaves of four contrasting species in order to establish a guideline for large-scale *in situ* RWC measurements. I used resealable plastic bags or microcentrifuge tubes to store the leaves during the transport. I assessed how long leaves could be stored for the measured RWC values to be accurate and how important it was to maintain cool temperatures during the

storage. Additionally, I tested the logistic feasibility of the method by measuring the daily course of leaf RWC of two species in a field situation.

5.3 Methods and Results:

Leaf collection sites and species used – Two experiments were conducted to test the reliability of RWC measurements taken when immediate measurement of leaf fresh mass is not possible. In both experiments, leaf samples were collected on the Laurentian University Campus (46º27'59"N 80º58'23" W) in Sudbury, Ontario Canada. Four species with contrasting leaf characteristics and vulnerability to desiccation were selected: *Impatiens capensis* Meerb. (Balsaminaceae) and *Scirpus microcarpus* J. Presl & C. Presl (Cyperaceae) are herbaceous species, whereas *Alnus incana* ssp. *rugosa* (Du Roi) R. T. Clausen (Betulaceae) and *Comptonia peregrina* (L.) J. M. Coulter (Myricaceae) are woody species. *I. capensis* occurs under shady and moist conditions and can be regarded as extremely poorly protected against desiccation, whereas *C. peregrina* grows on exposed rocky sites and is well protected against water loss. *A. incana* is a wetland shrub/small tree forming dense shady canopies, whereas *S microcarpus* grows in open but wet conditions. *I. capensis* and *C. peregrina* probably capture the extremes of desiccation vulnerability across the species at the collection site. Desiccation speed for the leaves of the four species was tested by laying them for one hour unprotected on a laboratory counter at about 27°C. During this hour, *I. capensis* lost 62±5% of its leaf fresh mass, *A. incana* 13±0%, *S. microcarpus* 10±2%. and *C. peregrina* 5±1% (mean±1SE; N=6).

Experiment 1 – In this experiment change in leaf fresh mass due to loss of water during storage in plastic bags or microcentrifuge tubes was documented over a period of up to 96 h. Branches or shoots from each of the four species were collected in a large plastic bag in the field on July 23, 2012, 9:00 a.m., and brought within 15 min to the laboratory, where they were placed in water-filled beakers. Twenty fresh leaves were collected per species, weighed and placed into pre-weighed resealable 10 ×15 cm 0.05 mm (2 mil) plastic polybags (WAT supplies, Sudbury, ON, Canada). For *C. peregrina*, the species with the smallest leaves, 1.5 mL Fisherbrand™ Premium microcentrifuge tubes were used. All the bags and tubes with leaves were weighed and the values recorded as values at time zero. The bags and tubes were then put in sealed plastic containers for storage. Half the samples were stored at room temperature in the dark, the average temperatures during the 4 days of the experiment being 28.7, 27.6, 27.0, 25.7 ºC, respectively. The other half of the bags were placed in a refrigerator with a temperature of 10.0 ºC. Temperatures were measured with iButton dataloggers (DS1921G; Maxim Integrated, San Jose, California). All bags with leaves were re-weighed after 3, 6, 24, 48 and 96 h after the first measurement. Leaf fresh mass at the time of measurement was calculated by deducting the bag mass from the measured mass of the bag with leaf. This value was then normalized by dividing it by the initial, pre-bagging leaf mass, enabling a direct comparison of relative fresh mass changes of all the leaves.

Leaves of all species lost water during the storage in plastic bags, and the loss was faster at room temperature than in the refrigerator (Tables 5.1 and Fig. 5.1). For *C. peregrina* leaves, the species best protected against desiccation and the only species stored in microcentrifuge tubes there was only a slight decrease in fresh mass over time, slightly faster under the warm storage temperature, but none of these effects was

significant. After 24 h in the refrigerator, *I. capensis* leaves had lost 7% of their fresh mass, but for the other three species the loss did not exceed 2%. At room temperature, fresh mass declined faster, but remained for all species, but *I. capensis,* within 94% of the original during the first 24 hours. Even after 96 h under the cool conditions, leaves of all species but *I. capensis* lost maximally 6%. For this species the loss was 17%. Under the warm conditions, *I. capensis* leaves lost 62% of their fresh mass within 96 h. For *C. peregrina* the loss was 1% and 9% within 96 hours under cold and warm conditions, respectively, the effects being non-significant.

Experiment 2 – In this experiment changes in leaf RWC, leaf fresh mass to saturated mass ratio, and leaf dry mass to saturated mass ratio were documented after storage in plastic bags or microcentrifuge tubes after 24 h and after 96 h, by comparing the values to the initial measurements. RWC is the target variable, which is calculated using leaf fresh mass, leaf saturated mass and leaf dry mass. The fresh mass to saturated mass, and the dry mass to saturated mass ratios indicate how the components of RWC contribute to its change.

As saturated mass and dry mass of the stored leaves could not be repeatedly measured, each time of measurement required a separate set of leaves. On July 24, 2012, 9:00 a.m. 240 leaves were harvested on campus. Sixty leaves from each of the four species were collected directly in resealable bags or microcentrifuge tubes (*C. peregrina*). The bagged samples were transported in a cooler to the laboratory within maximally one hour after collection. Two thirds of the bags containing the leaves were then stored in airtight plastic boxes either in a refrigerator (10.0 ºC) or at room temperature (25.0-28.5 ºC, average temperatures during the 4 days of the experiment being 27.6, 27.0, 25.7,

25.45 ºC, respectively). For one third of the leaves their fresh mass was immediately measured, for another third after 24 h storage, and for the last third of the leaves after 96 h storage. After the measurement of fresh mass, the leaves were placed in a refrigerator between moist paper towels for 24 h to attain saturation (Ryser *et al.,* 2008). Leaf saturated mass was measured, after which the leaves were dried at 75ºC for 48 h, and their dry mass determined.

Leaf relative water content decreased during prolonged storage for all species (Tables 5.1 and Fig. 5.2). The decline was faster under warm conditions. Under cool conditions, the RWC values lost after 24 h was maximally 5% of the initial values, and under warm conditions maximally 12%. After 96 h, RWC had further declined, except for warm-stored *A. incana* and *C. peregrina* leaves, for which RWC increased between 24 and 96 h. All the three factors: time, temperature and species, and their interactions were highly significant when data for all species were included into one ANOVA (analysis not shown). In specieswise ANOVA's time had a significant effect on RWC of all species but *C. peregrina*, the interaction with storage temperature being significant for *I. capensis* and *S. microcarpus* (Table 5.1). Changes in the fresh mass to saturated mass ratio were close to those of RWC, but dry mass to saturated mass ratio showed a different behavior (Fig. 5.2). In all species but *S. microcarpus* there was a significant Time × Temperature interaction (Table 5.1). *A. incana*, the ratio increased in the cold and the warm treatment already after 24 h. For *C. peregrina* an increase was observed in warm treatment only, for *I. capensis* in the cold treatment after 96 h. I am not aware of a mechanism to explain an increase of dry mass of leaves stored under cool and dark conditions, and the increased dry mass to saturated mass ratio seems to indicate of a

decreased ability of the leaves to absorb water under saturating conditions, probably due to changes at the cellular level.

Field measurements of RWC – To test the practicality of the method under a real field situation (46°37'52" N 81°14'38" W), diurnal fluctuations of leaf RWC were measured for two species, *Bidens cernua* (Asteraceae*)* and *Carex retrosa* (Cyperaceae) in a Sudbury area wetland. Leaves of these two species were collected on five occasions from dawn to dusk (6:30, 10:00, 14:00, 18:00, 20:00) on September 9, 2012, with 10 replicate leaves at a time into pre-weighed resealable plastic bags. The bagged samples were placed inside a cooler (7-10 ºC) and brought to the laboratory the following day. After the leaf fresh mass was measured, leaf saturated mass and dry mass were measured as described for Experiment 2.

Both species showed a significant diurnal variation in their RWC, declining from the first measurement at 6:30 until 18:00, but reaching close to initial values by 20:00 (Fig. 5.3). *C. retrorsa* had throughout lower values of RWC than *Bidens cernua*. Compared to the diurnal variation and interspecific differences the standard error was relatively small, and the effects of species ($p<0.001$) and time ($p<0.001$) were highly significant, but not their interaction ($R^2=0.467$, ANOVA, N=10, one outlier with a studentized residual >5 removed).

Table 5.1. Results of repeated measures ANOVA on leaf fresh mass (FM) with 6 measurements after storage of 0 h, 3 h, 6 h, 24 h, 48 h, and 96 h (Experiment 1, data arcsine transformed, one outlier removed) and results of ANOVAs of leaf relative water content (RWC) , leaf fresh mass to saturated mass ratio (FM/SM), and leaf dry mass to saturated mass ratio (DM/SM) measured after 0h, 24 h and 96 h storage in a refrigerator or at room-temperature with time of storage and storage temperature as factorial variables (Experiment 2, seven outliers removed). ANOVAs were conducted for each species separately. F values and the level of significance are given (*p<0.01; **p<0.01; *** p<0.001, n.s. not significant).

	N	R2	Temperature	Time	Temp x Time
FM, Experiment 1					
Impatiens capensis	20		65.7***	273***	57.3***
Alnus incana	19		142***	739***	131***
Comptonia peregrina	20		0.0 n.s.	1.2 n.s.	1.6 n.s.
Scirpus microcarpus	20		60.3***	159***	28.5***
RWC, Experiment 2					
Impatiens capensis	58	0.664	1.1 n.s.	73.4 ***	10.4 **
Alnus incana	59	0.179	0.8 n.s.	10.3. **	0.2 n.s.
Comptonia peregrina	58	0.029	1.0 n.s.	0.6 n.s.	0.9 n.s.
Scirpus microcarpus	58	0.561	4.9 *	40.9 ***	15.0 ***
FM/SM, Experiment 2					
Impatiens capensis	58	0.67	1.3 n.s.	73.9 ***	11.2 **
Alnus incana	59	0.141	1.0 n.s.	7.5 **	0.4 n.s.
Comptonia peregrina	58	0.04	1.6 n.s.	0.6 n.s.	1.6 n.s.
Scirpus microcarpus	58	0.552	5.0 *	40.3 ***	14.6 ***
DM/SM, Experiment 2					
Impatiens capensis	58	0.154	2.9 n.s.	2.9 n.s.	5.6 *
Alnus incana	59	0.286	2.6 n.s.	16.1 ***	4.5 *
Comptonia peregrina	58	0.159	7.6 **	0.2 n.s.	9.8 **
Scirpus microcarpus	58	0.055	1.1 n.s.	0.1 n.s.	0.2 n.s.

Fig. 5.1. Leaf fresh mass of the four investigated species after storage times from 3 to 96 h, stored in a refrigerator (light symbols) or at room temperature (dark symbols). The values are normalized to the directly measured initial mass. The error bars indicate ±1 SE.

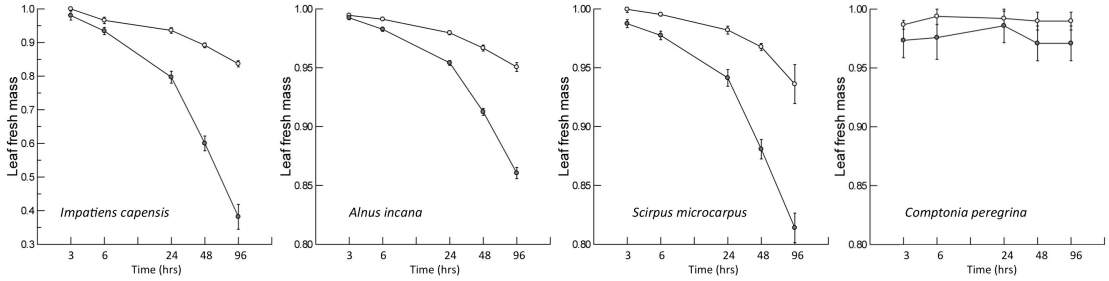
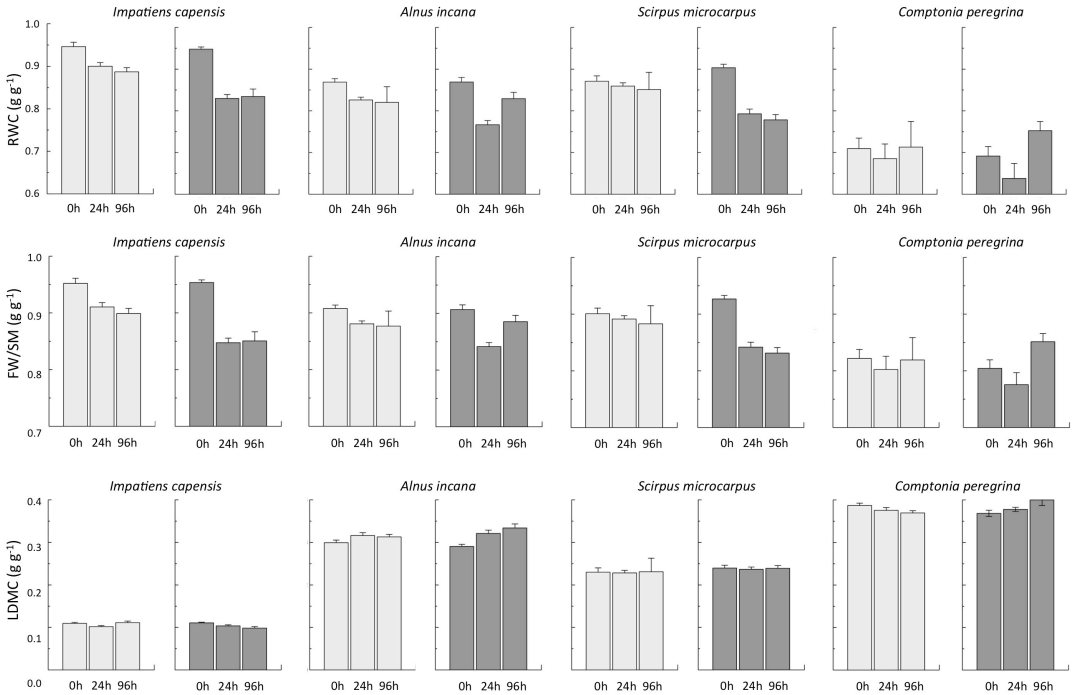


Fig. 5.2. Leaf relative water content (RWC), leaf fresh mass to saturated mass ratio (FM/SM), and leaf dry mass to saturated mass ratio (leaf dry matter content; LDMC) for the four investigated species, measured after 0h, 24 h and 96 h storage in a refrigerator (light grey bars) or at room-temperature (dark grey bars).



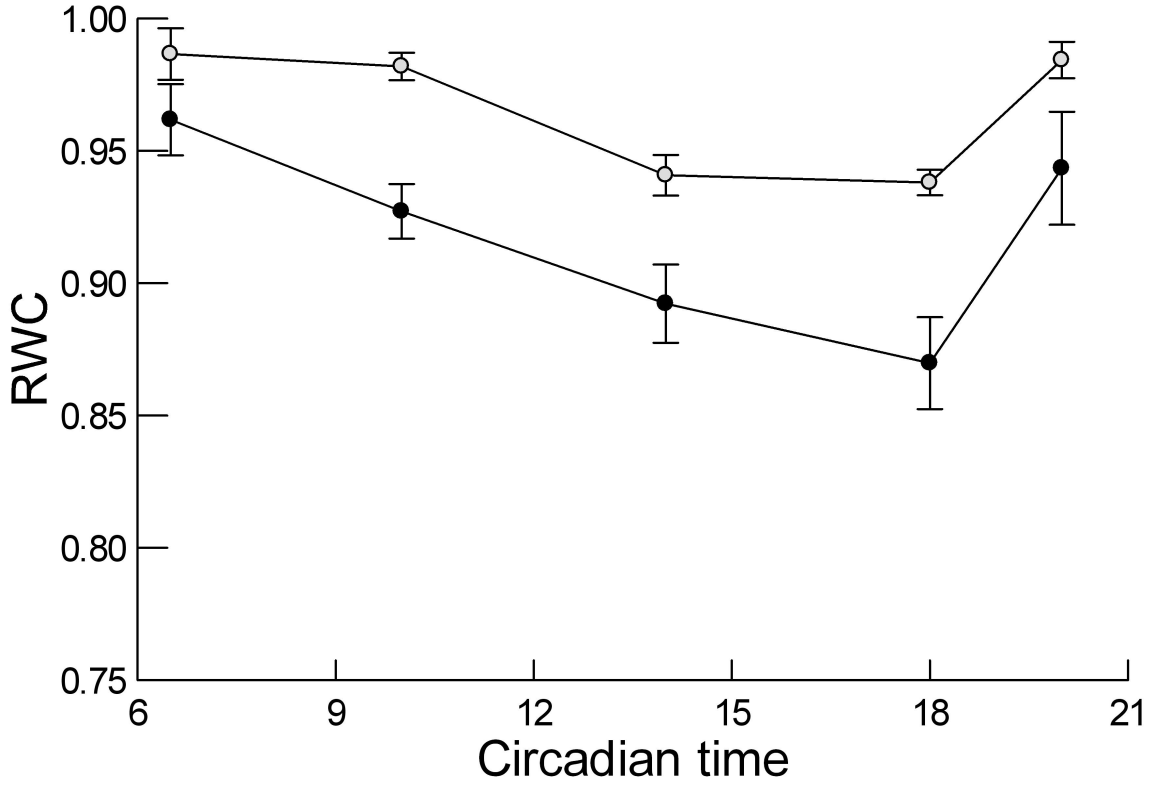


Fig. 5.3. Diurnal fluctuations in relative water content (%) of leaves of *Bidens cernua* and *Carex retrosa* on September 9, 2012, between 6:30 and 20:00 Eastern Daylight Time. N=10. Leaves were stored in a cooler until measurement in the laboratory the following day.

5.4 Conclusions:

Re-sealable plastic bags are frequently used to assess leaf water content in field-grown plants, but to our knowledge any potential measurement errors caused by water loss from leaves stored in such bags has previously not been quantified. Our results show that their use to transport leaves from the field to the laboratory allows determination of leaf RWC of field-grown plants with reasonable accuracy. When stored cool, the RWC remained within 5% of the initial value for at least 24 hours.

The extent of the storage-caused change in the calculated RWC, however, depends on the identity of the species. For species that are poorly protected against desiccation, such as *I. capensis* from wet and shady environments, loss of fresh mass was 7% even from a sealed plastic bag under cool conditions, whereas more desiccation-tolerant species, such as *C. peregrina* from dry and sunny habitats, were less influenced by a delay in the measurement, losing less water even after 96 hours. Hence, the constraints for the accuracy of the RWC measurement in the field depend on type of the investigated habitat. Notably, during storage the leaves not only lost water, but their ability to gain mass under saturating conditions declined as well, especially when stored at room temperature. This is an indication of changes at the cellular level, e.g., membrane degradation (Deschene *et al*., 1991), and the pronounced change in *A. incana* indicates that this effect is species specific as well.

Plastic bags are to some extent permeable to gases, and a slow diffusion of water molecules through plastic will lead to loss of water over time. Less permeable containers, such as metal and glass jars as used by early researchers (Stocker, 1929; Weatherley, 1950) avoid this problem, but large leaves require large containers and the required volume may become difficult to keep cool and to use in the field. Such containers also

are heavier than plastic bags, and their mass may exceed the capacity of high-resolution balances able to measure the leaf mass accurately. Removing leaves from the container at weighing may lead to further loss of water, especially if any has condensed in the walls of the container. For small leaves, such as conifer needles, microcentrifuge or polyethylene tubes can be used (Armas, 2010; Afer, 2013). Our data indicate a minimal water loss from the tubes, but it is not conclusive because leaves of the species used in the tubes was also the species with the best traits to protect against desiccation.

Methods using foliar reflectance to measure RWC have been developed (e.g., Chen *et al*., 2012) and deliver reliable results for RWC. However, the equipment is not necessarily widely available, and gravimetric determination of RWC remains an important way to assess plant water status.

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CONCLUSION AND RECOMMENDATIONS

A review of the current literature demonstrates that there is increasing evidence that heavy metal contamination strongly affects plant water relations **(Chapter 1)**. Progress during the last few years has been especially great in the area of understanding how metals affect plants at the molecular level, such as aquaporin function. However, actual research on how plants respond to simultaneous metal and drought stresses is rare, and the results contradictory, often varying with the metal that was used as a contaminant. For example, whereas exposure to slag containing Ni and Cu exacerbates the effects of drought (de Silva *et al.,* 2012), elevated levels of Zn alleviate them (Disante *et al.,* 2014). The experiments in the present thesis show that contamination of soil by Ni and Cu indeed exacerbates drought stress, with effects from the organ to ecosystem-level, and can increase mortality under drought.

In a two-year lysimeter study (**Chapter 2**) it was found that metals have a more detrimental effect on plant-water relationships then originally hypothesized. In the combined metal and drought treatment, transpiration was dramatically reduced, but at the same time, leaf Relative Water Content decreased. In some instances this led to increased leaf temperatures. The reduced water use increased available soil moisture in metal contaminated pots under drought compared to the drought-only treatments. These results indicate that an important mechanism of the additional drought stress imposed by metals is impeded water uptake. In contrast, there also was indication that drought stress may aggravate metal stress as drought resulted in increased leaf Cu concentrations.

Mechanisms leading to the impeded water uptake by metal-exposed plants were investigated in an experiment comparing the effects of long-term vs. short-term Ni^{2+}

exposure of *Betula papyrifera* plants (**Chapter 3**). A history of growth on contaminated substrate resulted in a reduced leaf area of plants, which was the main contributor towards lower transpiration. Short-term exposure to Hg reduced transpiration rates per leaf area within hours. There was some indication that damage caused by long-term exposure to metals may influence the short-term response of transpiration rates to Ni, but the results were inconclusive, probably due to confounding effects of size variation. However, Ni^{2+} has been shown to reduce membrane transport of water in roots (Llamas *et al*., 2008).

de Silva *et al.* (2012) and Lamoreaux and Chaney (1977) have shown that metal treatments, as do drought treatments, reduce xylem area and vessel size and hence hydraulic conductance. As small xylem vessel size is associated with hydraulic safety (Hacke *et al.,* 2006), this could mean that metals improve plants' drought tolerance. This actually has been shown to be the effect of Zn (Disante *et al.,* 2014). However, in this thesis (**Chapter 4**), saplings collected from a region of historical metal deposition had a lower embolism resistance compared to saplings with no contamination exposure, despite their lower hydraulic conductance. This is evidence that Cu and Ni contamination makes plants more vulnerable to drought, and may be a reason for the characteristic 'coppiced' appearance, stem dieback, and lower seedling establishment commonly found in metal contaminated regions (James and Courtin, 1987).

Overall, drought creates challenges for plants in metal-contaminated soils. As water uptake is inhibited, the level of soil moisture does not directly relate to plant stress, but a given level of drought is perceived to be more severe by the plants. Not only does metal contamination lead to lower RWC, but it makes them more prone to stem- and branch-dieback by embolism formation. Therefore, it is my recommendation that future

restoration projects select tree species that are drought tolerant. One may assume that these species have already adapted to drought that minimize both embolisms and maintain a more constant leaf relative water content during periods of drought. However, more comparative research is needed to understand interspecific differences in the effects of metals on plant-water relationships. As drought has been identified as having an additive effect on metal stress responses in plants, it is also my recommendation that restoration managers look for ways to increase the water-holding capacity of metal-contaminated soils, for example by increasing organic matter content (Hudson, 1994).

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